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Applicant	Keiya Ozawa et al.	Confirmation No.	2019
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Customer No.:	21559		
Title:	FUSION PROTEIN THAT IMPARTS SELECTIVE PROLIFERATION ACTIVITY		

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DECLARATION OF YASUJI UEDA UNDER 37 C.F.R. § 1.132

I declare:

1. I am a co-inventor of the subject matter that is described and claimed in the above-referenced application. I am currently an Assistant Professor at the Chiba University Graduate School of Medicine. I have over 20 years technical experience in the fields of Molecular Biology, Cell Biology, Immunology, etc. A copy of my curriculum vitae is attached as Appendix A.

2. G-CSF receptors are a class of proteins that were extraordinarily well characterized at the time the application was filed. The structural features, including the Immunoglobulin-like domain, the cytokine receptor homologous domain, the three fibronectin type III domains, and the intracellular domain, defining this class of receptors were also known. For instance, Fukunaga et al. (Cell 61:341-350, 1990; copy enclosed as Exhibit A) describes the murine G-CSF receptor sequence and notes that the sequence is highly homologous to that of the human G-CSF receptor. Larsen et al. (J. Exp. Med. 172:1559-1570, 1990; copy enclosed as Exbibit B) describes the human G-CSF receptor sequence. In addition, Fukunaga et al. (EMBO J.: 10:2855-2865, 1991;

copy enclosed as Exhibit C) describes functional domains of human and mouse G-CSF receptors. In view of the knowledge in the art at the time the application was filed, a skilled artisan would readily recognize a G-CSF receptor sequence.

3. Estrogen receptors are part of a conserved and well-characterized family of proteins. The sequences of various members of the estrogen receptor family were known at the time the application was filed. For instance, the human estrogen receptor sequence was disclosed in a 1986 publication (see Greene et al., Science 231:1150-1154, 1986; copy enclosed as Exhibit D). Given that the structure of the estrogen receptor and of its estrogen-binding domain was known at the time the application was filed, one skilled in the art would readily recognize whether a given sequence is that of an estrogen receptor. Moreover, a skilled artisan, at the time of filing, would have recognized an estrogen- binding domain of an estrogen receptor.

4. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

December 13, 2006
Date

Yasuji Ueda

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Immunology

List of publications:

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Expression Cloning of a Receptor for Murine Granulocyte Colony-Stimulating Factor

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Summary

Two cDNAs encoding the receptor for murine granulocyte colony-stimulating factor (G-CSF) were isolated from a CDM8 expression library of mouse myeloid leukemia NFS-60 cells, and their nucleotide sequences were determined. Murine G-CSF receptor expressed in COS cells could bind G-CSF with an affinity and specificity similar to that of the native receptor expressed by mouse NFS-60 cells. The amino acid sequence encoded by the cDNAs has demonstrated that murine G-CSF receptor is an 812 amino acid polypeptide (M_r 90,814) with a single transmembrane domain. The extracellular domain consists of 601 amino acids with a region of 220 amino acids that shows a remarkable similarity to rat prolactin receptor. The cytoplasmic domain of the G-CSF receptor shows a significant similarity with parts of the cytoplasmic domain of murine interleukin-4 receptor. A 3.7 kb mRNA coding for the G-CSF receptor could be detected in mouse myeloid leukemia NFS-60 and WEHI-3B D^+ cells as well as in bone marrow cells.

Introduction

Production of hematopoietic cells is regulated by hormone-like growth and differentiation factors called colony-stimulating factors (CSFs). CSFs include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin-3 (IL-3) (Metcalf, 1989; Nicola, 1989). G-CSF, produced mainly by macrophages, is important in regulating blood levels of neutrophils and in activating mature neutrophils. G-CSF stimulates some myeloid leukemia cells to proliferate or to differentiate into neutrophilic granulocytes (Nagata, 1990).

Murine G-CSF and human G-CSF have been purified (Nicola et al., 1983; Nomura et al., 1986), and cDNAs encoding G-CSF have been molecularly cloned (Nagata et al., 1986a, 1986b; Souza et al., 1986; Tsuchiya et al., 1986). Human G-CSF is a 174 amino acid polypeptide, while murine G-CSF consists of 178 amino acids. Human and mouse G-CSFs are highly homologous (72.6%) at the amino acid sequence level, in agreement with the lack of species specificity between them (Nicola et al., 1985). Although the primary structure of G-CSF does not show homology with other CSFs or growth factors, it has a weak similarity with interleukin-6 (IL-6), which stimulates proliferation and differentiation of B-lymphocytes (Nagata, 1990). Human G-CSF produced by recombinant DNA technology has proven to be a potent regulator of neutrophils in vivo using animal model systems (Tsuchiya et al., 1987; Nicola, 1989). Recent clinical trials in patients suffering from a variety of hemopoietic disorders have shown that the administration of G-CSF is beneficial in chemotherapy and bone marrow transplantation therapy (Morstyn et al., 1989).

Despite the biological importance of G-CSF, little is known about the mechanism of G-CSF-induced signal transduction in the proliferation and differentiation of neutrophilic granulocytes. Several reports in human and mouse systems have suggested that the expression of the G-CSF receptor is restricted to progenitor and mature neutrophils and various myeloid leukemia cells (Nicola and Metcalf, 1984, 1985; Nicola et al., 1985; Begley et al., 1987; Park et al., 1989). However, the G-CSF receptor has also recently been found in nonhemopoietic cells, such as human endothelial cells (Bussolino et al., 1989) and placenta (Uzumaki et al., 1989). Biochemical characterization of the G-CSF receptor has been hampered by the low number of receptors present on the cell surface (at most 1000–2000 receptors per cell). A limited number of studies have indicated that cells of the neutrophilic lineage have a single class of binding sites for G-CSF with an equilibrium dissociation constant of 100–500 pM (Nicola and Metcalf, 1984; Park et al., 1989; Uzumaki et al., 1989). Cross-linking studies of the receptor with the radiolabeled G-CSF have suggested a M_r of 150,000 for the mouse G-CSF receptor in WEHI-3B D^+ cells (Nicola and Peterson, 1986). Recently, we were able to solubilize mouse G-CSF receptor in an active form from NFS-60 cells and succeeded in purifying the receptor as a protein with a M_r of 100,000–130,000 (R. F., E. I., and S. N., unpublished data).

In this work, we isolated cDNAs encoding the murine G-CSF receptor from mouse myeloid leukemia NFS-60 cells. When transfected into COS cells, the cDNA directed expression of a receptor that has similar properties to that of the native G-CSF receptor on NFS-60 cells. The amino acid sequence of the G-CSF receptor indicates that it belongs to the recently identified growth factor receptor family (Bazan, 1989).

Results

Expression Cloning of the G-CSF Receptor cDNA

To isolate the cDNA coding for the G-CSF receptor, we used a COS cell expression system developed to isolate the murine erythropoietin receptor (D'Andrea et al., 1989). Double-stranded cDNA was synthesized using mRNA from mouse myeloid leukemia NFS-60 cells, which have relatively higher expression of the G-CSF receptor than other G-CSF-responsive myeloid leukemia cells, such as WEHI-3B D^+ or 32DC13. A cDNA library was constructed in the mammalian expression vector CDM8 (Seed, 1987) as 884 pools of 60–80 clones. Plasmid DNAs from each

pool were prepared by the boiling method and introduced into COS-7 cells grown in 6-well microtiter plates. At 72 hr posttransfection, binding reactions of ^{125}I-G-CSF (1.7×10^5 cpm [200 pM] in a 0.6 ml volume) to COS cells were carried out at 37°C for 2 hr instead of 4°C in order to obtain a greater signal (D'Andrea et al., 1989). Under these conditions, the background binding of labeled G-CSF to transfected or untransfected COS cells was routinely 308 ± 38 (SD) cpm. Plasmid DNAs from two pools (I62 and J17) yielded binding of 500 cpm and 912 cpm of ^{125}I-G-CSF, respectively, when transfected into COS-7 cells. The bacterial clones of pools I62 and J17 were arranged in 12 subgroups of 12 clones each and assayed as above. Some subgroups gave positive responses, that is, binding of 3710–4010 cpm of ^{125}I-G-CSF to COS cells. By assaying single clones from each positive subgroup, two independent clones (pI62 and pJ17) were identified. When plasmid DNAs from pI62 and pJ17 were transfected into COS-7 cells, the binding assay gave values of 30,300 cpm and 31,600 cpm, respectively.

Binding Characteristics of the Cloned Receptor

The binding characteristics of the G-CSF receptor expressed on COS cells were examined. COS cells transfected with the plasmid CDM8 or pJ17 were incubated at 4°C for 4 hr with various concentrations of ^{125}I-G-CSF in the presence or absence of at least a 500-fold excess of unlabeled G-CSF (800 nM). Untransfected COS cells or COS cells transfected with the CDM8 vector alone did not show any significant specific binding of ^{125}I-G-CSF. On the other hand, labeled G-CSF was bound at 4°C to the COS cells transfected with the plasmid pJ17. As shown in Figure 1, a Scatchard analysis of the specific binding of ^{125}I-G-CSF to COS cells revealed a single species of binding site with an equilibrium dissociation constant of 290 pM and 3.0×10^4 receptors per cell. If the transfection efficiency of COS cells was assumed to be 10%–20% (Somparyrac and Danna, 1981), the positively transfected COS cells probably expressed the recombinant G-CSF receptor at $1.5–3.0 \times 10^5$ molecules per cell. Since the native G-CSF receptor on NFS-60 cells has an equilibrium dissociation constant of 180 pM (Figure 1D), these results suggest that the polypeptide coded by the cDNA in the plasmid pJ17 is sufficient to express the high-affinity receptor for murine G-CSF.

Human G-CSF competes with mouse G-CSF for binding to mouse WEHI-3B D$^+$ cells (Nicola et al., 1985). Accordingly, an excess of unlabeled recombinant human G-CSFs produced either by mammalian cells or Escherichia coli could compete well with labeled mouse G-CSF for binding to COS cells transfected with the plasmid pJ17 (Figure 2). No inhibition of binding of ^{125}I-G-CSF to COS-7 cells was observed in the presence of unlabeled recombinant murine GM-CSF, murine IL-3, murine IL-6, murine leukemia inhibitory factor (LIF), rat prolactin, or human M-CSF. These results correlated well with the specificity of the native G-CSF receptor or the purified receptor on NFS-60 cells (R. F., E. I., and S. N., unpublished data).

Previously, we observed that the G-CSF receptor purified from NFS-60 cells has a M_r of 100,000–130,000. To

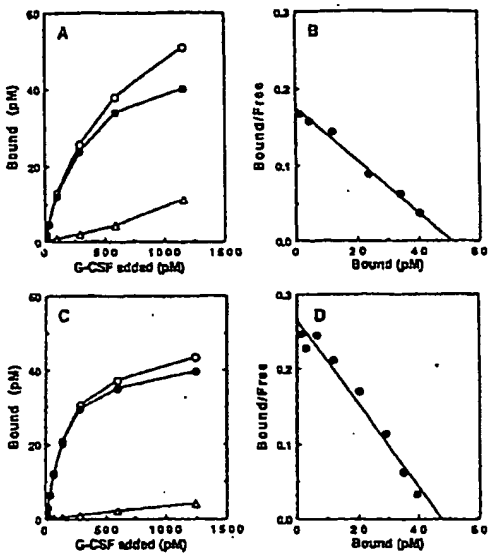

Figure 1. Binding of Radioiodinated G-CSF to COS Cells Expressing the Recombinant G-CSF Receptor and NFS-60 Cells

(A) Saturation binding of ^{125}I-G-CSF to COS cells. COS cells (1×10^6) transfected with the plasmid pJ17 were incubated with various amounts of ^{125}I-G-CSF with or without an excess of unlabeled G-CSF as described in the Experimental Procedures. The specific binding (●) is shown as the difference between total (○) and nonspecific (△) binding.

(B) Scatchard plot of G-CSF binding data in COS cells.

(C) Saturation binding of ^{125}I-G-CSF to NFS-60 cells. Total (○), nonspecific (△), and specific (●) binding to cells are shown.

(D) Scatchard plot of G-CSF binding data in NFS-60 cells.

determine the molecular size of the recombinant G-CSF receptor expressed in COS cells, chemical cross-linking of the receptor with ^{125}I-G-CSF was carried out. As shown in Figure 3, cross-linking of the G-CSF receptor on NFS-60 cells with labeled mouse G-CSF (M_r 25,000) yielded a band with an apparent M_r of 125,000–155,000 (lane 6), indicating that the M_r of the murine G-CSF receptor on NFS-60 cells is 100,000–130,000. Similarly, cross-linking of ^{125}I-mouse G-CSF to the receptor expressed in COS cells gave a major band of M_r 120,000–150,000 (lane 4), which is slightly smaller than that detected in NFS-60 cells. These bands were not observed when the cross-linking experiment was carried out in the presence of 1.5 μM unlabeled G-CSF (lanes 2 and 5) or when the cross-linking agents were omitted (lane 3). The slightly different M_r observed in COS cells and NFS-60 cells may be explained by the differential glycosylation in these cell lines.

The Structure of Murine G-CSF Receptor

Digestions of the plasmid pJ17 and pI62 with XhoI released cDNA inserts of 3.2 kb and 3.0 kb, respectively. As shown in Figure 4A, the restriction maps of these inserts were identical except that the 5′ terminus of clone pI62 is 81 bp longer than that of pJ17 and the 3′ terminus of the clone pJ17 is 238 bp longer than that of pI62. When

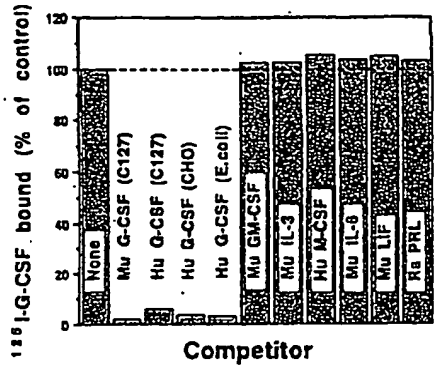

Figure 2. Specificity of G-CSF Binding to Recombinant G-CSF Receptor Expressed in COS Cells

COS cells transfected with the cDNA for the G-CSF receptor (pJ17) were incubated with 2 ng of ^{125}I-mouse G-CSF in the absence or presence of 1 μg of unlabeled murine G-CSF, human G-CSF, murine GM-CSF, human M-CSF, murine IL-6, murine LIF, or rat prolactin. As human G-CSF, human recombinant G-CSFs produced in mouse C127 cells, in Chinese hamster ovary cells, or in E. coli were used. The radioactivities bound to COS cells in each experiment are expressed as a percentage of that obtained without competitor.

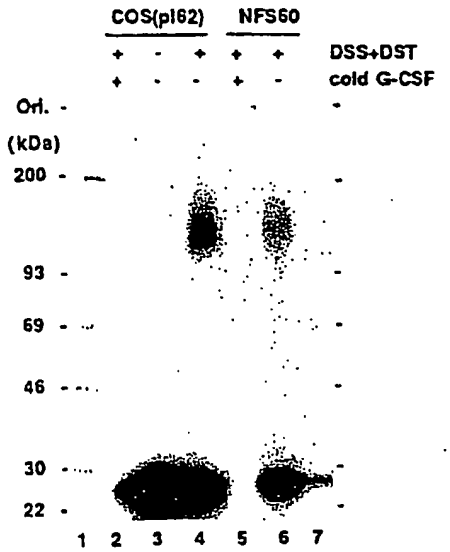

Figure 3. Chemical Cross-Linking of the G-CSF Receptor Expressed in COS and NFS-60 Cells

COS cells (5.2 × 10^5 cells/lane) transfected with the plasmid pI62 were incubated with ^{125}I-G-CSF with (lane 2) or without (lanes 3 and 4) an excess of unlabeled murine G-CSF and chemically cross-linked as described in the Experimental Procedures. Mouse NFS-60 cells (3 × 10^6 cells/lane) were similarly incubated with ^{125}I-G-CSF with (lane 5) or without (lane 6) an excess of unlabeled G-CSF and cross-linked with DSS and DST. The cell lysate was analyzed by SDS–PAGE on a 4%–20% gradient polyacrylamide gel and exposed to X-ray film at −80°C for 2 days with intensifying screens. As size markers, ^{14}C-labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel (lanes 1 and 7), and sizes of standard proteins are shown in kd.

the cDNAs were sequenced, the two sequences were found to be identical within the overlapping region. Although the two cDNAs contained the complete coding sequence for the G-CSF receptor, they contained neither the poly(A) tract nor the poly(A) addition signal. The cDNA library was, therefore, rescreened by colony hybridization using the 2.5 kb HindIII–XbaI fragment of pJ17 as a probe. Fifteen positive clones were obtained from about 60,000 clones, and one of them (pF1) had 603 bp of 3′ noncoding region and contained two overlapping poly(A) addition signals. The composite nucleotide sequence of the three cloned cDNAs (pI62, pJ17, and pF1) is presented in Figure 5 together with the predicted amino acid sequence. There is a long open reading frame starting from the initiation codon ATG at nucleotide positions 180–182 and ending at the termination codon TAG at positions 2691–2693. The open reading frame (2511 nucleotides) can code for a protein consisting of 837 amino acids, including the NH_2-terminal methionine. In the 5′ sequence upstream of the long open reading frame, three other potential initiation codon ATGs can be found at positions 73, 105, and 126. All of these are followed by short open reading frames. Deletion of these ATG codons from the cDNA by digesting the plasmid pI62 with HindIII did not increase or decrease the expression level of the recombinant G-CSF receptor in COS cells (R. F. and S. N., unpublished data).

The long open reading frame starts with a stretch of hydrophobic amino acids that seems to serve as a signal se-

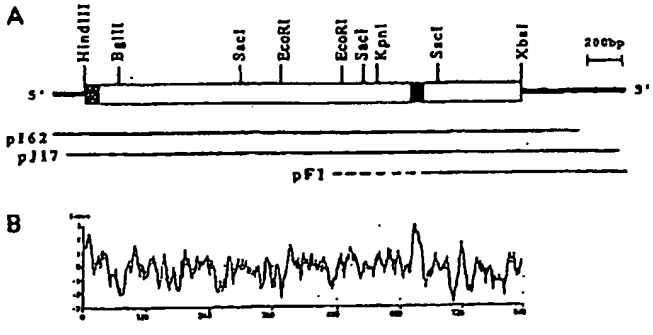

Figure 4. Murine G-CSF Receptor cDNAs

(A) Schematic representation and restriction map of three independent cDNAs (pI62, pJ17, and pF1) for murine G-CSF receptor. The box represents the open reading frame. The stippled and black regions indicate the signal sequence and the transmembrane region, respectively. The cleavage sites for restriction enzymes are shown.

(B) Hydropathy plot of the amino acid sequence of murine G-CSF receptor. The hydropathy plot was obtained by the method of Kyte and Doolittle (1982) using a window of ten residues. The numbers under the plot indicate positions of the amino acid residues of the precursor protein.

quence. By comparing the 5' portion of the sequence with typical signal peptide cleavage sites (von Heijne, 1986), the 26th amino acid (Cys) from the initiation codon was tentatively assigned as the first amino acid of the mature protein. The mature murine G-CSF receptor thus would consist of 812 amino acids with a calculated M_r of 90,814, which is 5,000–35,000 smaller than the M_r (95,000–125,000) estimated from the cross-linking experiment (Figure 3) or the M_r of the purified murine G-CSF receptor (R. F., E. I., and S. N., unpublished data). The difference is probably due to the attachment of sugar moieties to some of the 11 putative N-glycosylation sites (Asn-X-Thr/Ser) found on the extracellular domain of the G-CSF receptor (Figure 5). A hydropathy plot (Kyte and Doolittle, 1982) of the amino acid sequence of the mature G-CSF receptor (Figure 4B) revealed a stretch of 24 uncharged amino acids extending from Leu-602 to Cys-625, which is followed by three basic amino acids. These properties are consistent with those observed in the membrane-spanning segments of many proteins (Sabatini et al., 1982). The mature G-CSF receptor thus appears to consist of an extracellular domain of 601 amino acids, a membrane-spanning domain of 24 amino acids, and a cytoplasmic domain of 187 amino acids. The NH_2-terminal half of the extracellular domain is abundant in cysteine residues (17 residues in 373 amino acids), which seems to be a feature common to the ligand-binding domain of many receptors (McDonald et al., 1989). As found in the erythropoietin receptor (D'Andrea et al., 1989), the G-CSF receptor is rich in proline (80 residues, 9.9%). Furthermore, the content of tryptophan residues in murine G-CSF receptor is relatively high (26 residues, 3.2%), although they show no particular area of localization within the receptor.

Expression of the G-CSF Receptor mRNA

G-CSF stimulates the proliferation of mouse myeloid leukemia NFS-60 cells, while WEHI-3B D^+ cells can be induced to differentiate into monocytes and granulocytes by G-CSF (Nagata, 1990). To determine whether the same mRNA is expressed in NFS-60 and WEHI-3B D^+ cells, Northern hybridization was carried out using the cDNA from plasmid pJ17. As shown in Figure 6, a 3.7 kb mRNA could be detected in RNAs from NFS-60 cells (lanes 2 and 3) as well as from WEHI-3B D^+ cells (lane 5). The amount of mRNA for the G-CSF receptor is about ten times higher in NFS-60 cells than in WEHI-3B D^+ cells, which agrees with our observation that NFS-60 cells bind three to four times more ^{125}I-G-CSF than WEHI-3B D^+ cells (unpublished data). In contrast, no transcript for the G-CSF receptor was detected in RNAs from other mouse myeloid leukemia FDC-P1 cells (lane 4), which do not respond to G-CSF, or from nonhemopoietic cell lines such as L929 (lane 1) or C127I (data not shown). When mRNA expression was examined in various mouse tissues, only bone marrow cells gave a signal corresponding to the 3.7 kb mRNA (lane 9). The similar size of the mRNAs observed in bone marrow cells and NFS-60 cells suggests that the authentic mRNA for the G-CSF receptor is expressed in mouse myeloid leukemia NFS-60 cells.

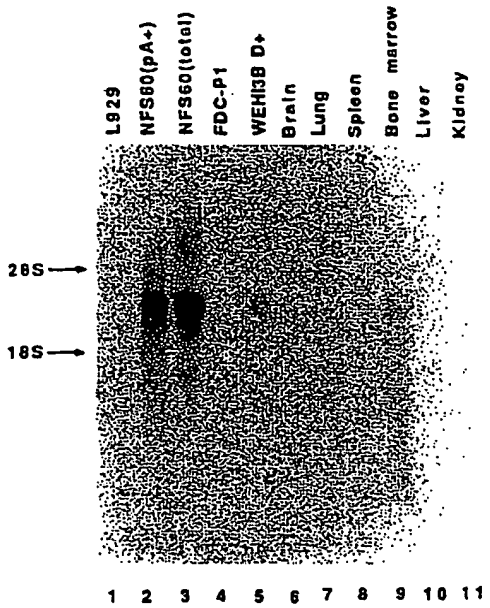

Figure 6. Northern Hybridization Analysis of Murine G-CSF Receptor mRNA

Total RNA or poly(A) RNA was prepared from mouse cell lines: L929 (lane 1), NFS-60 (lanes 2 and 3), FDC-P1 (lane 4), and WEHI-3B D^+ (lane 5), or from mouse tissues: brain (lane 6), lung (lane 7), spleen (lane 8), bone marrow (lane 9), liver (lane 10), and kidney (lane 11). Total RNA (30 μg) (lanes 1 and 3–11) or 2 μg of poly(A) RNA (lane 2) was electrophoresed on a 1.3% agarose gel containing 6.6% formaldehyde and analyzed by Northern hybridization as described in the Experimental Procedures.

Discussion

The mechanisms of signal transduction induced by various hemopoietic growth and differentiation factors, including G-CSF, are not fully understood, in part because their receptors are expressed in low levels on the cell surface. Recently, receptors for several cytokines and lymphokines have been molecularly cloned by various techniques (Yamasaki et al., 1988; Mosley et al., 1989; Hatakeyama et al., 1989; D'Andrea et al., 1989; Gearing et al., 1989; Itoh et al., 1990). To isolate the cDNA for the G-CSF receptor, we used the expression cloning method developed by D'Andrea et al. (1989). We chose this method since a single

Figure 5. Nucleotide Sequence and Predicted Amino Acid Sequence of the Murine G-CSF Receptor cDNA

Numbers above and below each line refer to the nucleotide position and amino acid position, respectively. Amino acids are numbered starting at Cys-1 of the mature G-CSF receptor. On the amino acid sequence, the signal sequence and the transmembrane domain are underlined. Two overlapping poly(A) addition signals (AATAAA) are also underlined. Potential N-glycosylation sites (Asn-X-Ser/Thr) (11 in the extracellular domain and 2 in the cytoplasmic domain) are boxed.

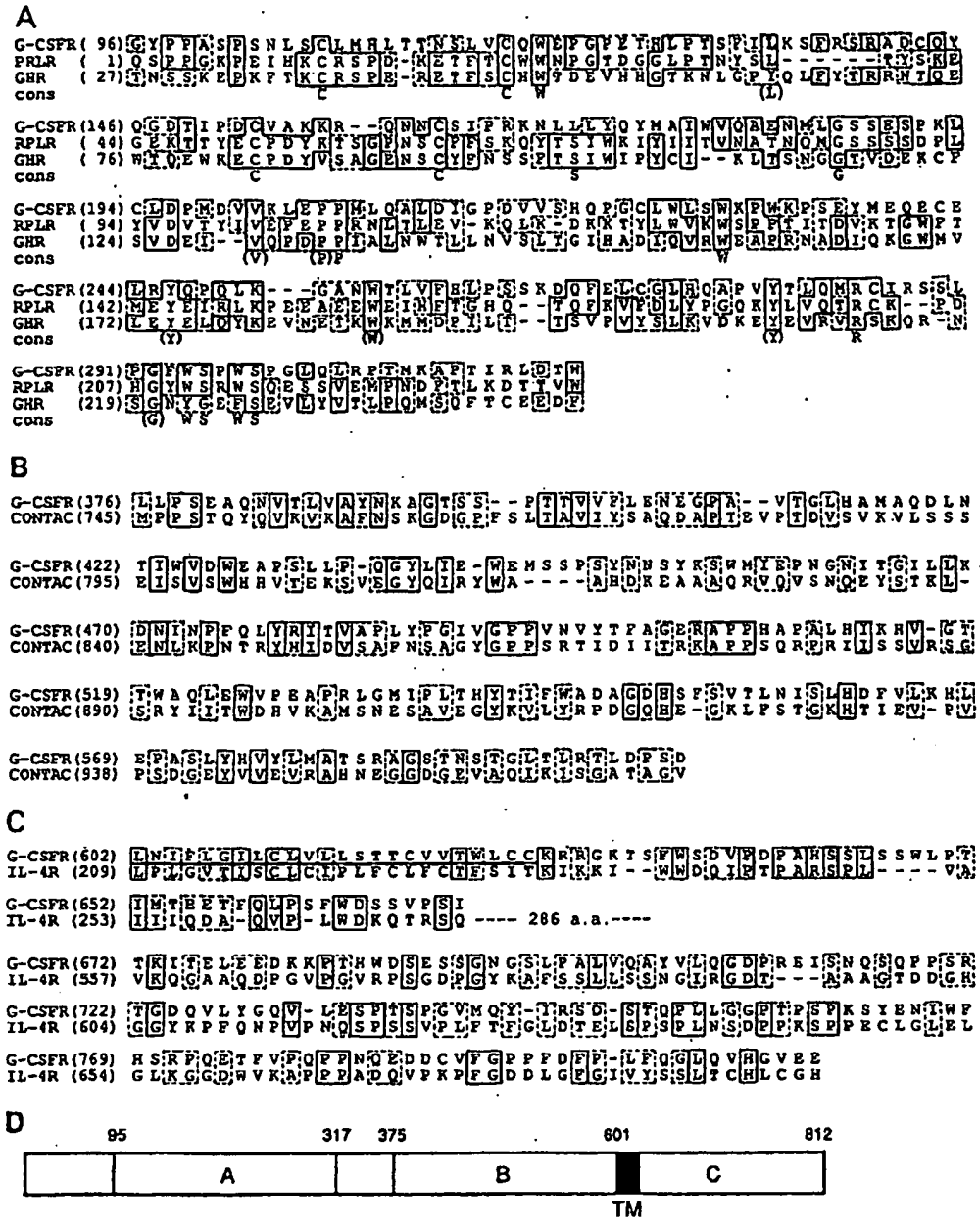

Figure 7. Comparison of the Amino Acid Sequence of the G-CSF Receptor with That of Other Growth Factor Receptors

(A) Alignment of the G-CSF receptor with prolactin and growth hormone receptors. The amino acid sequence from 96 to 317 of the murine G-CSF receptor is aligned with rat prolactin and human growth hormone receptors to give maximum homology by introducing several gaps (–). Identical residues in two sequences are enclosed by solid lines, and residues regarded as favored substitutions are enclosed by broken lines. Favored amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W. Amino acids conserved in nine members of the growth factor receptor family (G-CSF, prolactin, growth hormone, erythropoietin, GM-CSF, IL-2β, IL-3, IL-4, and IL-6) are shown under each line with or without brackets. The residues without brackets are conserved in more than eight members, and the residues with brackets are conserved in five to seven members in the family.

(B) Alignment of the G-CSF receptor with contactin. The amino acid sequence from 376 to 601 of the mouse G-CSF receptor is aligned with the amino acid sequence of chicken contactin as described in (A).

polypeptide purified from NFS-60 cells is sufficient to bind G-CSF with a high affinity (R. F., E. I., and S. N., unpublished data) and little background binding of G-CSF to COS cells is observed.

Expression of the cloned cDNA gave rise to a protein that shows the same binding properties as those of the G-CSF receptor in mouse NFS-60 cells. Recombinant and native G-CSF receptors could bind ^{125}I-G-CSF with a dissociation constant of 200–300 pM (Figure 1), could specifically bind mouse G-CSF or human G-CSF, but could not bind other growth and differentiation factors such as IL-3, GM-CSF, M-CSF, IL-6, LIF, and prolactin (Figure 2). Furthermore, the cross-linking experiment indicated that the M_r of the recombinant G-CSF receptor (100,000–130,000) expressed in COS cells is similar to that of the G-CSF receptor purified from NFS-60 cells or the polypeptide cross-linked in NFS-60 cells (Figure 3). These results indicate that the G-CSF binding protein in NFS-60 cells is coded by the cloned cDNA. However, upon stimulation by G-CSF, it is not known whether this polypeptide alone can transduce the signal into cells or whether other proteins are required to form a functional receptor capable of conferring G-CSF responsiveness.

The amino acid sequence of murine G-CSF receptor (Figure 5) has many properties commonly found in the receptors for growth and differentiation factors (McDonald et al., 1989). It has a signal sequence at the N-terminal and a single transmembrane domain, suggesting that the N-terminal part of the molecule is extracellular and the C-terminal part remains inside the cell. Within the first 80 amino acids of the mature G-CSF receptor are four cysteines, two of which (Cys-21 and Cys-77) are organized in a manner characteristic of receptors of the immunoglobulin superfamily (Williams and Barclay, 1988). However, the sequence between these two cysteine residues does not seem to fulfill the criteria for recognition as a member of the immunoglobulin superfamily (Williams and Barclay, 1988).

Comparison of the amino acid sequence of the G-CSF receptor with all sequences in the National Biomedical Research Foundation data base revealed that one part of the extracellular domain of the G-CSF receptor has remarkable similarities with the prolactin receptor and another part has remarkable similarities with contactin (Figure 7). Prolactin is an anterior pituitary hormone and belongs to the family consisting of growth hormone, prolactin, and placental lactogen (Cooke et al., 1981). The amino acid sequences of human prolactin and growth hormone show a homology of 48% when conservative changes in amino acids are included. Accordingly, as shown in Figure 7A, the entire extracellular domain of the prolactin receptor (210 amino acids; Boutin et al., 1988) has a similarity of 43.2% (60 identical amino acids and 38 homologous amino acids) with a domain of the growth hormone receptor (Leung et al., 1987). To some extent, growth hormone competes with prolactin in binding to the prolactin receptor (Boutin et al., 1988) and vice versa (Leung et al., 1987). When the amino acid sequence from 96 to 317 of the mouse G-CSF receptor was aligned with the extracellular domain of the rat prolactin receptor, 54 of 227 amino acids were identical and 40 more represented conservative substitutions, yielding an overall similarity of 41.4%. The regions homologous between G-CSF and prolactin receptors are not well conserved in the growth hormone receptor, resulting in a low similarity (34.4%) between G-CSF and growth hormone receptors. Despite the similarity in amino acid sequence of the extracellular domains of the G-CSF and prolactin receptors, a 500-fold excess of unlabeled rat prolactin did not inhibit the binding of ^{125}I-G-CSF to the recombinant G-CSF receptor expressed in COS cells (Figure 2). This is consistent with the fact that the amino acid sequence of G-CSF has no significant homology to that of prolactin. These results may suggest that regions of the extracellular domain of the G-CSF receptor that are not similar to the prolactin receptor are required for the binding of G-CSF. In this regard, it is notable that the extracellular domain of the G-CSF receptor is 391 amino acids larger than that of the prolactin receptor.

When the sequences of the ligand binding domains of growth factor receptors were compiled, it was suggested that the receptors for growth hormone, prolactin, erythropoietin, and IL-6, as well as for the β chain of the IL-2 receptor, belong to a novel receptor family (Bazan, 1989). Recently isolated receptors for IL-4 (Mosley et al., 1989), IL-3 (Itoh et al., 1990), and GM-CSF (Gearing et al., 1989) are also members of this receptor family. The consensus amino acids in the family are indicated in Figure 7A. In the G-CSF receptor, the consensus cysteine and tryptophan residues are conserved, and the "WSXWS" motif (Gearing et al., 1989; Itoh et al., 1990) is also found at amino acid residues 294–298; this suggests that the G-CSF receptor belongs to the family. In this comparison of the G-CSF receptor with other hemopoietic growth factor receptors, it may be noteworthy that the similarity of the G-CSF and IL-6 receptors is less pronounced than that of the G-CSF and prolactin receptors, although G-CSF and IL-6 have a similarity of 44.6% (Nagata, 1990).

As shown in Figure 7B, the amino acid sequence from 376 to 601 in the extracellular domain of the G-CSF receptor has a significant similarity (42.9%) with a part of the extracellular domain of chicken contactin (Ranscht, 1988). Contactin is a neuronal cell surface glycoprotein of 130 kd and seems to be involved in cellular communication in the nervous system. Because the region from amino acid

(C) Alignment of the G-CSF receptor with the IL-4 receptor. The amino acid sequence from 602 to 808 of the mouse G-CSF receptor is aligned with two corresponding regions of mouse IL-4 receptor as above.

(D) Schematic representation of the mouse G-CSF receptor. The box indicates the mature G-CSF receptor. "TM" represents the transmembrane domain. Region "A" indicates a domain (222 amino acids) with similarity to other growth factor receptors, including prolactin and growth hormone receptors, and contains the "WSXWS" motif. Region "B" (226 amino acids) of the mouse G-CSF receptor shows similarity to chicken contactin. Region "C" (211 amino acids) includes the transmembrane domain (underlined) and the cytoplasmic domain of the G-CSF receptor and is similar to two regions of the mouse IL-4 receptor.

residues 737–818 of contactin can be aligned with the fibronectin type III segment involving binding to cells, heparin, and DNA, it is possible that this region plays an important role in cell adhesion (Ranscht, 1988). Granulopoiesis occurs daily in bone marrow, and the direct interaction of the neutrophilic progenitor cells with the bone marrow stroma cells has been proposed (Roberts et al., 1988). The similarity of part of the extracellular domain of the G-CSF receptor with contactin may suggest that this region is involved in the communication of neutrophilic progenitor cells and stroma cells.

The cytoplasmic domain consists of 187 amino acids and does not show any homology with the catalytic domain of the protein kinase family (Hanks et al., 1988). As observed in other growth factor receptors (Hatakeyama et al., 1989; Mosley et al., 1989), this region is rich in serine (12.8%) and proline (12.3%). When the transmembrane and cytoplasmic domains of the G-CSF receptor were aligned with the amino acid sequences of other growth factor receptors, a significant similarity with the IL-4 receptor was found. As shown in Figure 7C, the transmembrane domain and the first 46 amino acids of the cytoplasmic domain of the G-CSF receptor are homologous (50.0%) to the corresponding regions of the murine IL-4 receptor. Furthermore, amino acid residues 672–808 of the G-CSF receptor show significant similarity (45.4%) with amino acid residues 557–694 of the IL-4 receptor. These results suggest that signal transduction by G-CSF and IL-4 may be mediated by a similar mechanism.

The 3.7 kb mRNA for the G-CSF receptor was detected not only in NFS-60 cells but also in WEHI-3B D$^+$ cells (Figure 6), suggesting that the same G-CSF receptor is involved in G-CSF-induced proliferation of NFS-60 cells and differentiation of WEHI-3B D$^+$ cells. The different effects of G-CSF on NFS-60 and WEHI-3B D$^+$ cells may therefore be mediated by different signal transduction mechanisms downstream of the receptor. In this regard, it is interesting that the *c-myb* and *evi-1* loci, which appear to be involved in differentiation of myeloid cells, are rearranged in NFS-60 cells but not in WEHI-3B D$^+$ cells (Morishita et al., 1988). When RNAs from several mouse tissues were examined, the transcript for the G-CSF receptor was detected only in bone marrow cells that contain the progenitor for neutrophilic granulocytes. However, since G-CSF has some effect on bone remodeling (M. Y. Lee, R. F., T. J. Lee, J. L. Lottsfeldt, and S. N., submitted) and growth of endothelial cells (Bussolino et al., 1989), a low-level expression of the G-CSF receptor in other tissues cannot be ruled out. Under low-stringency hybridization, mRNA for the human G-CSF receptor could be detected in some human myeloid leukemia cells (R. F., Y. S., and S. N., unpublished data) using mouse G-CSF receptor cDNA as a probe. Availability of cDNA for the human G-CSF receptor would be valuable in the screening of various leukemia cells from human patients for the expression of the G-CSF receptor before treatment of the patients with G-CSF (Morstyn et al., 1989). Furthermore, the soluble form of the G-CSF receptor may be useful clinically to inhibit the proliferation of some human myeloid leukemia cells that are dependent upon G-CSF (Santoli et al., 1987).

Experimental Procedures

Cells

Mouse myeloid leukemia NFS-60 cells (Weinstein et al., 1986; kindly provided by J. Ihle, St. Jude Children's Research Hospital) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10–20 U/ml of recombinant mouse IL-3. COS-7 cells were routinely maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Recombinant Colony-Stimulating Factors

Human recombinant G-CSF was purified from medium conditioned with mouse C1271 cells, which were transformed with the bovine papillomavirus expression vector (Fukunaga et al., 1984) carrying human G-CSF cDNA (Tsuchiya et al., 1987). Mouse G-CSF was produced by using a similar expression system and purified to homogeneity (R. F., E. I., and S. N., unpublished data). Human recombinant G-CSF and M-CSF produced by Chinese hamster ovary cells were provided by Chugai Pharmaceutical Co. Human recombinant G-CSF produced by E. coli was purchased from Amersham. Mouse recombinant IL-3 and GM-CSF were generous gifts from Dr. A. Miyajima and K. Arai (DNAX Institute). Mouse recombinant IL-6 and mouse recombinant LIF were generously provided by Dr. T. Hirano (Osaka University) and N. Nicola (Walter Eliza Hall Institute), respectively. Rat prolactin was purchased from Chemicon International, Inc.

Mouse recombinant G-CSF was radioiodinated by the IODO-GEN method (Fraker and Speck, 1978) with a slight modification (R. F., E. I., and S. N., unpublished data). Specific radioactivities ranged from 6–8 × 10^6 cpm/ng protein (1200–1600 cpm/fmol).

Construction of the CDM8 cDNA Library

Total RNA was prepared from exponentially growing NFS-60 cells by the guanidine isothiocyanate/CsCl method (Chirgwin et al., 1979), and poly(A) RNA was selected by oligo(dT)-cellulose column chromatography. Double-stranded cDNA was synthesized as described (Nagata et al., 1986a) using a kit from Amersham, except for the reverse transcriptase, which was purchased from Seikagaku Kogyo Co. Addition of BstXI nonpalindromic linkers to the blunt-end cDNA and size fractionation of cDNA on a 1% agarose gel were performed using a kit from Invitrogen. cDNA larger than 1.9 kb was recovered from the gel and ligated to BstXI-digested CDM8 vector (Seed, 1987). E. coli MC1061/p3 cells were transformed with the ligated DNA by the electroporation method as described (Dower et al., 1988).

DNA Preparation

A total of 6 × 10^4 bacterial colonies were plated on agar at a density of 60–80 colonies per well using 24-well microtiter plates, and glycerol cultures for each pool of colonies were prepared. LB broth was inoculated with aliquots from each glycerol culture, and plasmid DNAs were prepared by the boiling method (Maniatis et al., 1982) followed by phenol extraction and ethanol precipitation.

Transfection of COS-7 Cells

Monolayers of COS-7 cells were grown in 6-well microtiter plates, and transfection of plasmid DNA into COS-7 cells was carried out by a modification of the DEAE-dextran method (Sompayrac and Danna, 1981). In brief, about 50% confluent cells were washed three times with serum-free DMEM and incubated for 8 hr at 37°C with 0.6 ml of DMEM containing 50 mM Tris–HCl (pH 7.3), 0.3 mg/ml DEAE-dextran, and 1 μg of plasmid DNA. After glycerol shock with Tris–HCl-buffered saline containing 20% glycerol for 2 min at room temperature, cells were washed twice with DMEM and incubated in DMEM containing 10% FCS.

Screening of Transfectants of COS-7 Cells

At 72 hr after transfection, COS-7 cells were washed with DMEM containing 10% FCS and 20 mM HEPES (pH 7.3) (binding medium) and incubated at 37°C for 2 hr with 1.7 × 10^5 cpm (200 pM) of ^{125}I-G-CSF in 0.6 ml of the binding medium. Unbound radioiodinated G-CSF was removed, and cells were successively washed three times with phosphate-buffered saline (PBS) supplemented with 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and once with PBS. Cells were then recovered by trypsinization, and the radioactivity associated with cells was counted using an AUTO-GAMMA 5000 MINAXI γ-counter (Packard). Background

binding of ^{125}I-G-CSF to COS-7 cells transfected with the CDM8 vector was 308 ± 38 (SD) cpm. Two positive pools were identified that showed significant binding of radioiodinated G-CSF (500 and 912 cpm) to the transfected COS-7 cells. Independent clones (144) from each positive pool were grown in six 24-well microtiter plates and subjected to sib selection (Maniatis et al., 1982) using a matrix of 12 × 12 clones. After a final round of minipreparation of plasmids and transfection into COS-7 cells, a single clone was identified from each positive pool.

Binding of ^{125}I-G-CSF to COS Cells and NFS-60 Cells

COS cells grown on 15 cm plates were transfected with 20 μg of the pI62 or pJ17 plasmid as described above except that cells were treated for 3 hr with a DEAE-dextran solution containing DNA. Cells were split into 6-well microtiter plates 12 hr after the glycerol shock and grown for 60 hr in DMEM containing 10% FCS. Cells were washed with binding medium and incubated at 4°C for 4 hr with ^{125}I-G-CSF (10 pM to 1.2 nM range) in 1.0 ml of the binding medium. To determine the nonspecific binding of ^{125}I-G-CSF to cells, a large excess of unlabeled G-CSF (800 nM) was included in the assay mixture, and the radioactivity bound to the cells was subtracted from the total binding to yield the specific binding. For binding of G-CSF to NFS-60 cells, 5.2 × 10^6 cells were incubated at 4°C for 4 hr with various concentrations of ^{125}I-G-CSF in 0.3 ml of RPMI-1640 medium containing 10% FCS and 20 mM HEPES (pH 7.3).

Chemical Cross-Linking

The chemical cross-linking of ^{125}I-G-CSF to the receptor expressed in COS cells was performed according to the procedure described for NFS-60 cells (R. F., E. I., and S. N., unpublished data). In brief, 8 × 10^5 of COS cells (on 3.5 cm plate) transfected with the plasmid pI62 were incubated at 4°C for 2.5 hr with 1.2 nM of the radioiodinated G-CSF in the presence or absence of 1.5 μM unlabeled G-CSF in 0.6 ml of the binding medium. The cells were scraped from the plate using a cell lifter and washed with 1 ml of PBS three times. Cross-linking was carried out on ice for 20 min in 1 ml of PBS containing 150 μM disuccinimidyl suberate (DSS) and 150 μM disuccinimidyl tartarate (DST). The reaction was stopped by the addition of 50 μl of 1 M Tris–HCl (pH 7.4), and cells were collected by centrifugation and lysed with 15 μl of 1% Triton X-100 containing a mixture of protease inhibitors (2 mM EDTA, 2 mM (p-aminophenyl)methanesulfonyl fluoride hydrochloride, 2 mM O-phenanthroline, 0.1 mM leupeptin, 1 μg/ml pepstatin A, and 100 U/ml aprotinin). After centrifugation, the clear lysate (10 μl) was analyzed by electrophoresis on a 4%–20% gradient polyacrylamide gel in the presence of SDS (Laemmli, 1970).

Hybridization and Nucleotide Sequence Analysis

Colony hybridization and Northern hybridization were carried out as described (Maniatis et al., 1982). As a probe, the 2.5 kb HindIII–XbaI fragment of clone pJ17 was labeled with ^{32}P by the random primer labeling method (Feinberg and Vogelstein, 1983).

DNA sequencing was performed by the dideoxynucleotide chain termination method using T7-DNA polymerase (Pharmacia) and [α-^{35}S]-dATPαS (Amersham).

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Expression Cloning of a Human Granulocyte Colony-stimulating Factor Receptor: A Structural Mosaic of Hematopoietin Receptor, Immunoglobulin, and Fibronectin Domains

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Summary

We report the isolation from a placental library, of two cDNAs that can encode high affinity receptors for granulocyte colony-stimulating factor (G-CSF) when expressed in COS-7 cells. The cDNAs are predicted to encode integral membrane proteins of 759 and 812 amino acids in length. The predicted extracellular and membrane spanning sequences of the two clones are identical, as are the first 96 amino acids of their respective cytoplasmic regions. Different COOH termini of 34 or 87 residues are predicted for the two cDNAs, due apparently to alternate splicing. The receptor with the longer cytoplasmic domain is the closest human homologue of the murine G-CSF receptor recently described by Fukunaga et al. (Fukunaga, R., E. Ishizaka-Ikeda, Y. Seto, and S. Nagata. 1990. *Cell.* 61:341). A hybridization probe derived from the placental G-CSF receptor cDNA detects a ~3-kb transcript in RNAs isolated from placenta and a number of lymphoid and myeloid cells. The extracellular region of the G-CSF receptors is composed of four distinct types of structural domains, previously recognized in other cell surface proteins. In addition to the two domains of the HP receptor family-defining region (Patthy, L. 1990. *Cell.* 61:13) it incorporates one NH_2-terminal Ig-like domain, and three additional repeats of fibronectin type III–like domains. The presence of both an NH_2-terminal Ig-like domain and multiple membrane-proximal FN3-like domains suggests that the G-CSF receptor may be derived from an ancestral NCAM-like molecule and that the G-CSF receptor may function in some adhesion or recognition events at the cell surface in addition to the binding of G-CSF.

Granulocyte colony-stimulating factor (G-CSF)[1] is a glycoprotein secreted by macrophages, fibroblasts, and endothelial cells originally identified by its ability to stimulate the survival, proliferation, and differentiation in vitro of predominantly neutrophilic granulocytes from bone marrow progenitors (1). The capacity of G-CSF to regulate in vivo granulopoiesis is supported by animal and clinical studies, which demonstrated a reversible rise in circulating neutrophil levels in response to administered recombinant G-CSF (2). G-CSF has pleiotropic effects on mature neutrophils, enhancing their survival and stimulating functional activation, including induction of neutrophil alkaline phosphatase (3) and high affinity IgA Fc receptors (4), priming for respiratory burst (5, 6), and increased chemotaxis (7). G-CSF effects have also been observed on hematopoietic cells that are not committed to the granulocyte lineage, for example, stimulation of the proliferation or monocytic differentiation in vitro of some myeloid leukemic cells (8–10) and, in synergy with other colony-stimulating factors, the proliferation in vitro of some multipotential hematopoietic precursors (11–13). A recent clinical study implicates G-CSF in the regulation of human erythropoiesis (14). G-CSF may also affect nonhematopoietic cells, since it appears to stimulate the proliferation and migration of endothelial cells (15), and the growth of cell lines derived from colon adenocarcinomas (16) and small cell lung carcinomas (17).

These diverse effects of G-CSF are mediated by the interaction of G-CSF and specific cell surface receptors. Initial binding studies with native murine G-CSF detected low numbers of receptors on responsive murine cell lines and human bone marrow cells of the neutrophilic lineage (18–20). Affinity crosslinking studies suggested a murine receptor mo-

[1] *Abbreviations used in this paper:* FN3, fibronectin type III homology unit; G-CSF, granulocyte colony-stimulating factor; HP receptor, hematopoietin receptor; NCAM, neural cell adhesion molecule; PRL, prolactin.

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lecular weight (M_r) of ~150,000 (21). G-CSF muteins with improved stability have been shown to bind a single class of sites (K_d = 100–500 pM) on circulating neutrophils (22), U937 cells (23), placental membranes, and trophoblasts (24). Similar affinities have been measured for the binding of native G-CSF to a single class of sites on myeloid leukemic and small cell lung carcinoma cell lines (17). Although affinity crosslinking experiments detected human receptors of M_r ~150,000 on neutrophils, an additional crosslink to a protein of M_r ~120,000 could be detected on placental membranes, suggesting a more complex receptor composition (22, 24).

Here we report the isolation from a placental library of two cDNA clones that encode high affinity receptors for G-CSF when expressed in COS-7 cells. The two clones encode identical extracellular and transmembrane sequences, but differ in the COOH-terminal portion of their cytoplasmic regions, due to what appears to be alternate splicing. The predicted protein sequence of one clone suggests it is the strict human homologue of a recently cloned murine G-CSF receptor cDNA (25).

Materials and Methods

Human G-CSF Preparation. Human G-CSF (26) was expressed in yeast, using the α-factor secretion system (27), as a mutein in which Cys17 was replaced by serine and Arg22, by lysine. These alterations inhibit, respectively, the formation of disulfide-linked oligomers and inappropriate processing through destruction of a KexII recognition site. Alternatively, a fusion polypeptide of the same construct but incorporating a hydrophilic octapeptide at the NH_2 terminus to aid in purification (28) was also expressed. The biological activities of both purified forms were ~2×10^7 U/mg determined in a standard proliferation assay using the murine myeloid leukemic cell line DA-1. 1 U corresponds to that amount of G-CSF that gives half-maximal [^{3}H]TdR incorporation.

Radiolabeling of G-CSF. Purified human G-CSF was radiolabeled to a specific activity of 7×10^{14} cpm/mmol using a solid-phase chloramine-T analogue. 5 μg of purified G-CSF and 2 μCi $Na^{125}I$ in 150 μl PBS was placed in a 10 × 75 mm glass tube previously coated with 5 μg of Iodogen (Pierce Chemical Co., Rockford, IL) and incubated for 25 min, 4°C. Free and ligand-bound iodine were subsequently separated by gel filtration through a 1 ml column of Biogel P-6 (BioRad Laboratories, Richmond, CA) that had been blocked with BSA. Radiolabeled stocks were stored at 4°C in RPMI-1640 containing 2.5% BSA, 20 mM Hepes buffer, and 0.2% sodium azide, pH 7.2 (binding medium). The specific radioactivities of labeled G-CSF were based on determination of initial protein concentration by amino acid analysis, with correction from control experiments to determine protein recovery after iodination, in which an aliquot of G-CSF was mixed with ^{125}I–G-CSF and the iodination protocol repeated, with omission of $Na^{125}I$.

Cell Lines and Tissue Preparations. HL60, U937, C10, KG-1, HeLa, RAJI, MJ, and RPMI 1788 cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY), 10% FCS, 2 mM glutamine, and 50 μM 2-ME at 37°C in a humidified atmosphere of 5% CO_2 in air. Peripheral blood granulocytes were isolated by sedimentation on discontinuous Ficoll Hypaque (Sigma Chemical Co., St. Louis, MO) gradients followed by red blood cell lysis in ammonium chloride. Placental membranes were isolated as follows. Fresh, full-term human placenta, obtained from Swedish Hospital Medical Center (Seattle, WA) on ice, were washed six times with ice-cold PBS to extract excess blood, and the tissue, trimmed of amnion and chorion, was cut into small pieces. The pieces were transferred to one volume of Buffer A (30 mM Hepes, pH 7.4, containing 0.25 M sucrose, 1 mM PMSF, 1 μM pepstatin A, 10 μM leupeptin, 2 mM o-phenanthroline, and 0.02 U/ml aprotinin), homogenized for five 1-min intervals with a PT10/35 homogenizer (Brinkman, Westbury, NY) at setting 7 and centrifuged at 6,800 *g* for 30 min, 4°C. The supernatant was made 0.1 M in sodium chloride and 0.2 mM in magnesium chloride and centrifuged at 42,000 *g* for 40 min at 4°C. The resulting pellets were washed twice in Buffer A by resuspension and centrifugation as above. The final sedimented membranes were resuspended in 30 ml of Buffer A at a protein concentration of 10–20 mg/ml and stored at −70°C.

Binding Assays and Data Analysis. For equilibrium binding assays with native (placental membrane) G-CSF receptor, serial dilutions of ^{125}I–G-CSF in binding media were incubated with 300 μg membrane (protein) in 10 × 75 mm glass tubes in a total volume of 100 μl for 2 h, 4°C. Control experiments showed equilibration had been reached in this time. Bound ligand was measured by subsequent collection of membranes in the reaction mixture on glass microfiber filters (Whatman, Hillsboro, OR) using a vacuum filtration apparatus. Filters were washed three times with ice-cold PBS/BSA (1 mg/ml) before gamma counting. Nonspecific binding was determined for each data point with a control tube containing a 400-fold molar excess of unlabeled G-CSF. Free radiolabeled ligand for each data point was measured by counting an aliquot of ligand identically incubated in the absence of membranes, after subtraction of the corresponding bound counts. Binding curves were plotted in the Scatchard coordinate system, expressing bound ligand in units of fmole/milligram membrane protein.

For equilibrium binding assays with recombinant G-CSF receptor, COS-7 cells transfected with either the D-7 or 25-1 G-CSF receptor cDNA clone (COS-G-CSFr) were first diluted 10-fold with carrier cells (EL4-3$^+$ murine T cells) to prevent COS cell aggregation. EL4-3$^+$ and untransfected COS cells were both shown to lack receptors for human G-CSF. Serial dilutions of ^{125}I–G-CSF in binding media were incubated with cells (2×10^6 total cells/ml) for 2 h at 4°C in a total volume of 150 μl using 96-well microtiter plates. Free and bound ligand were separated by centrifugation of duplicate 60-μl aliquots of the reaction mixture in plastic tubes containing a phthalate oil mixture (29). The tubes were cut, and supernatant (free ligand) and pellets (bound ligand) were gamma counted. Nonspecific binding was determined by inclusion of a 200-fold molar excess of unlabeled G-CSF in the reaction mixture at one ligand dilution; the linearly extrapolated nonspecific binding was subtracted from each data point to generate specific binding. Binding parameters determined on adherent COS-G-CSF receptor cells were similar to those determined in the suspension assay.

Affinity Cross-linking. Adherent COS cells on 10-cm culture dishes transiently expressing the recombinant G-CSF receptor were incubated with ^{125}I–G-CSF (1 nM) in RPMI 1640 for 2 h at 4°C in the presence or absence of unlabeled G-CSF (1 μM). Cells were washed twice in ice-cold PBS and then crosslinked in situ with 0.1 mg/ml bis-(sulfosuccinimidyl) suberate (BS^3, Pierce Chemical Co.) in PBS at 25°C for 30 min. Cells were subsequently washed twice with PBS and then lysed with 0.5 ml of PBS/1% Triton containing protease inhibitors (2 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 2 mM o-phenanthroline, 2 mM EGTA, 1.25 mM benzamidine, 0.5 mM EDTA, and 2 μg/ml soybean trypsin inhibitor). Lysates were scraped from plates, microfuged at 12,000 *g* for 10 min, and supernatants retained. Placental membranes (8 mg pro-

tein/ml) were incubated in 1.5-ml plastic microfuge tubes with 1 mM ^{125}I-G-CSF in a total volume of 100 μl PBS for 2 h at 4°C in the presence or absence of unlabeled G-CSF (1 μM). Membranes were then washed two times with ice cold PBS, resuspended in 100 μl of PBS, and incubated with BS^3 (0.1 mg/ml) for 30 min at 25°C. Membranes were washed twice, then lysed in 150 μl of PBS 1% Triton (with protease inhibitors) for 30 min at 4°C. Insoluble debris was removed by centrifugation for 30 min at 10,000 *g*, and the supernatant was retained.

SDS-PAGE. Samples, including methyl ^{14}C-labeled molecular weight markers (Bethesda Research Laboratories, Bethesda, MD), were boiled for 30 min in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME) and analyzed on an 8% SDS gel (30). After electrophoresis, gels were fixed in 25% isopropanol, 10% acetic acid, dried, and autoradiographed with Kodak X-Omat AR film at −70°C.

cDNA Library Construction and Screening. Total cell RNA was isolated from whole fresh placental tissue as described below and polyadenylated RNA prepared by chromatography on oligo(dT)-cellulose as described (31). Double-stranded, oligo(dT)-primed cDNA was prepared with a commercial kit (Amersham Corp., Arlington Heights, IL). The resulting cDNA was size fractionated by chromatography on Sephacryl S-1000 (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.5 M sodium acetate. The excluded cDNA was cloned into the BglII site of the mammalian expression vector, pDC302 (32) by an adaptor method similar to that described by Haymerle et al. (33). Briefly, noncomplementary oligonucleotides of the sequence 5′-GATCTTGGAACGAGACGACCTGCT and 5′-AGCAGGTCGTCTCGTTCCAA synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA) were annealed and ligated in separate reactions to either cDNA or BglII cut vector. Nonligated oligonucleotides were separated from cDNA or vector by chromatography over Sepharose CL-2B (Pharmacia Fine Chemicals) at 65°C in 10 mM Tris (pH 8.0), 0.1 mM EDTA. 5 ng of adaptored vector was ligated to adaptored cDNA in 10-μl reactions containing 50 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 1 mM spermidine, 0.5 mM ATP, 0.1 U/μl T4 polynucleotide kinase and 0.4 U/μl T4 DNA ligase for 30 min at 37°C. Reactions were then desalted by drop dialysis on VSWP 013 filters (Millipore Corp., Bedford, MA) against distilled water for 40 min immediately before electroporation into *Escherichia coli* strain DH5 α as described (34). Transformants were obtained with an average cDNA insert size of 1.6 kb. Pools of 600 colonies were prepared and DNA minipreps of these were transfected into COS cells as described (35). After 3 d growth in DME/10% FCS, the cells were screened for ^{125}I-G-CSF binding by an in situ autoradiographic plate binding assay (36).

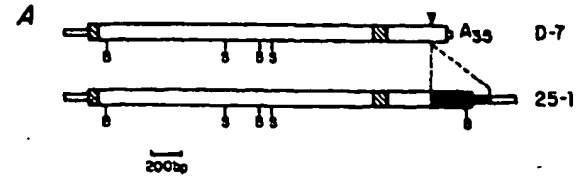
RNA Analysis. Total cellular RNAs were isolated by the guanidinium isothiocyanate–cesium chloride method and electrophoresed through formaldehyde agarose gels as described (31). RNA was transferred to nylon filters (Amersham) by capillary blotting and UV crosslinked using a Stratalinker (Stratagene, La Jolla, CA). Filters were probed with a ^{32}P-labeled antisense RNA prepared by T7 RNA polymerase transcription of a subclone of the D7 cDNA in pBlueSK (Stratagene). High stringency blot hybridization and washing conditions were as previously described (35).

Sequence Analysis. Sequences were aligned using various computer programs (GAP; 37, 38) and the progressive alignment method of Feng and Doolittle (39) as well as by visual inspection. With the exception of the alignment between the human and murine G-CSF receptors, a consensus alignment was generated for all sequences, rather than optimizing the alignment between any given pair of sequences. Alignment scores were generated using the NBRF program ALIGN using the MD data matrix with a bias of +6 and a gap penalty of 6. The prediction of residues involved in β-strands in immunoglobulin domain folding patterns used the turn and secondary structure prediction algorithms of Cohen et al. (40), the hydrophobic moment algorithm of Eisenberg et al. (41) as well as by inspection.

Results

Isolation of Human G-CSF Receptor cDNAs. Quantitative binding studies using radioiodinated G-CSF on a panel of human cell lines demonstrated low level expression of a single class of binding sites (N <1,000/cell, K_d ~1 nM; data not shown). G-CSF receptors with a similar affinity were detected on placental membranes (see below), but at a level of ~200 fmol/mg. Since binding of epidermal growth factor to A431 cell membranes at this level would correspond to a site number of ~10^6/cell (42) we concluded that G-CSF receptors were expressed at unusually high levels in placental tissue. A placental cDNA library was prepared in a mammalian expression vector and DNA from pools of ~600 transformants were transfected into COS cell that were then screened for ^{125}I-G-CSF binding by contact autoradiography (37). A positive clone, D7, was obtained after screening 20 pools and contained a 2.6-kb cDNA insert that was used as a hybridization probe to identify three additional related clones from the same library. Restriction digests and DNA sequencing showed that the cDNA clones fell into two classes: three were of the D7 type and one of a somewhat different form, 25-1, shown in Fig. 1. The 25-1 clone differs from the D7 clones only in its lack of a poly(A) tract and in the presence of a 419-bp internal sequence insert. This insert occurs between nt 2411 and 2412 of the D7 cDNA and appears to be derived from an unspliced intron since it contains splice donor and acceptor consensus sequences at the junctions with the D7 sequence.

DNA sequencing of these clones showed that the first ATG occurs in a context corresponding well to the Kozak consensus sequence (CCA/GCCATG; 43) and initiates a reading frame that terminates after 11 codons. The next potential initiation codon occurs 45 nt downstream, within an inferior Kozak context. This reading frame encodes proteins of 783 and 836 amino acids in the D7 and 25-1 cDNAs, respectively. Hydropathy analysis identified two major hydrophobic regions in the sequence (Fig. 1 D). The first, at the NH_2 terminus, is a presumed hydrophobic signal sequence of 24 residues; the second, between residues 604 and 629, is a presumed transmembrane domain that makes a single helical span (Fig. 1 *B*). Both forms of receptor are thus composed of an extracellular region of 603 amino acids and a transmembrane region of 26 amino acids, but differ in the predicted COOH-terminal portions of their cytoplasmic domains. The protein encoded by cDNA D7 has a cytoplasmic domain of 130 amino acids, while the unspliced intron sequence inserted in clone 25-1 after amino acid 725 predicts a cytoplasmic domain of 183 residues. The COOH-terminal amino acid sequence of D7 appears significantly more hydrophobic than that of 25-1 and contains one less Cys residue (Fig. 1). Both the D7 and 25-1 cytoplasmic sequences have high contents of proline (14.6 and



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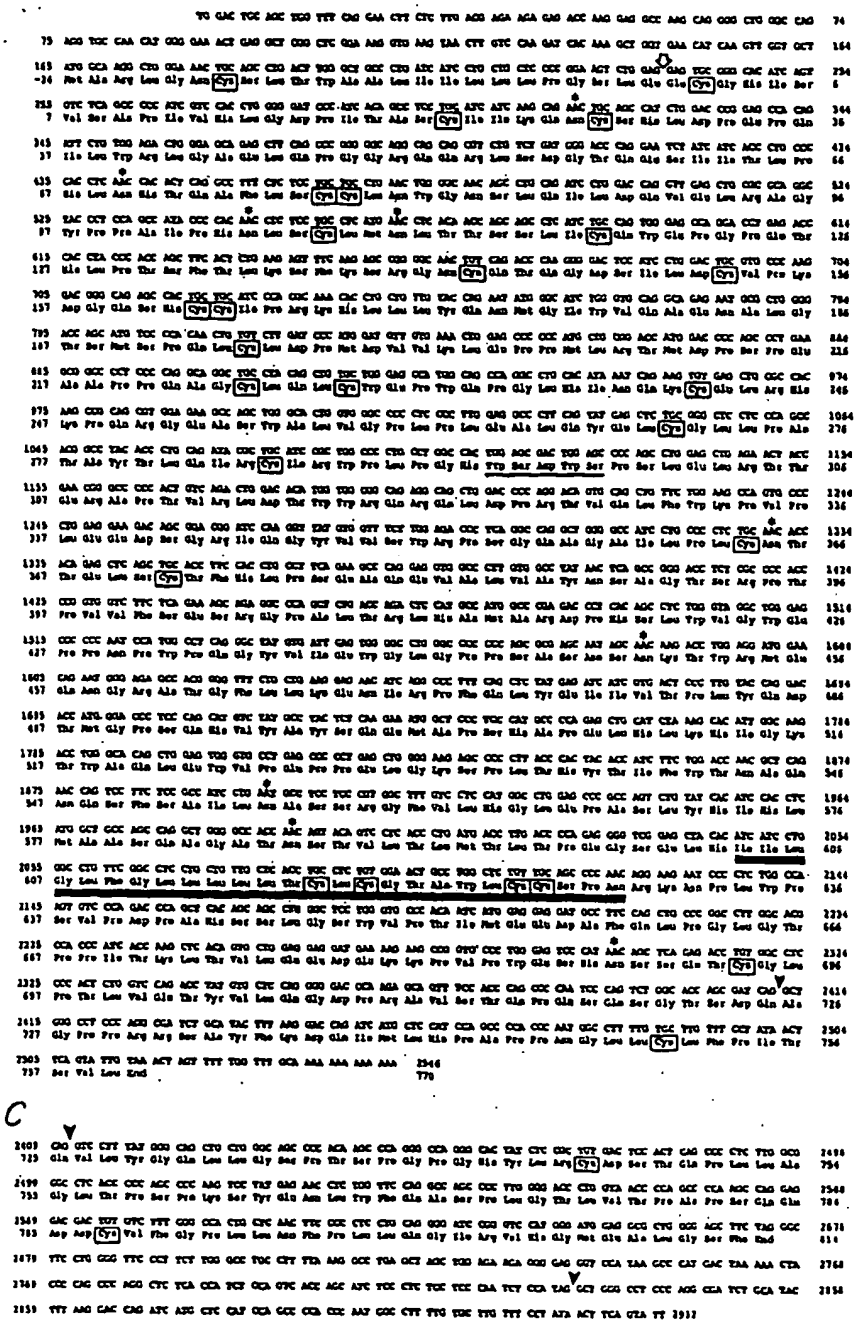

C

Figure 1. Human G-CSF receptor cDNAs. (*A*) Schematic representation and restriction map of G-CSF receptor cDNA clones D7 and 25-1. Restriction sites are indicated for BamHI (*B*) and SstI (*S*). The solid arrow marks the position in the D7 sequence at which the 25-1–specific insertion occurs. The insert sequence present only in the 25-1 clone is indicated as a filled bar, all other sequence is identical in the two clones. The deduced coding sequences are shown as as wide bars, noncoding sequences as narrow bars. The predicted signal transfer and transmembrane sequences are shown crosshatched. The 35-residue poly(A) tail of the D7 clone is shown as A35. (*B*) The nucleotide and deduced amino acid sequence of clone D7. The signal peptide cleavage site predicted by the probability weight matrix of von Heijne (75) is shown by an open arrow, and the predicted NH_2 terminus of the mature protein is designated residue 1. The predicted membrane spanning sequence is indicated by heavy underline and the Trp-Ser motif by a light underline. Cysteine residues are boxed and potential N-linked glycosylation sites are indicated by asterisks. The position of the 25-1 intron insertion point is indicated by a solid arrow. (*C*) The nucleotide and deduced amino acid sequence of the 3′ end of clone 25-1. Enumeration is continuous with *B*. Cysteine residues are boxed. Solid arrows indicate the junctions of the 419-bp insert with sequences common to both 25-1 and D7. (*D*) *On facing page.* Hydropathicity plot of the D7 and 25-1 receptor sequence according to the method of Kyte and Doolittle (76). The predicted signal and membrane-spanning sequences are indicated by arrows. These sequence data are available from EMBL/Genbank/DDJB under the accession numbers X55720 (clone D7) and X55721 (clone 25-1).

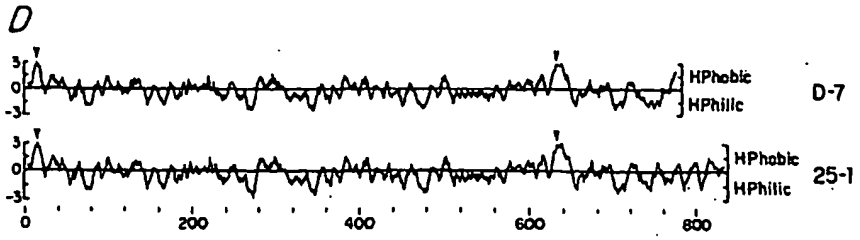

Figure 1 D.

13.1%, respectively) and serine (13 and 10.4%) a property noted for the cytoplasmic domains of many members of the hematopoietin (HP) receptor family (44). Neither the D7 nor 25-1 cytoplasmic regions contain sequences indicative of tyrosine kinase activity (45) but Ser760 of the 25-1 receptor represents a potential protein kinase C phosphorylation site (46). The predicted sequences of both G-CSF receptors contain nine potential N-linked glycosylation sites (Fig. 1 *B*), all but one in the proposed extracellular region. A murine G-CSF receptor cDNA isolated from myeloid leukemia cell library (25) encodes a predicted mature protein of 812 amino acids, identical in length to that of 25-1, and its COOH-terminal sequence is homologous to that of 25-1 but to D7 only up to the position of the proposed splice site. The 25-1

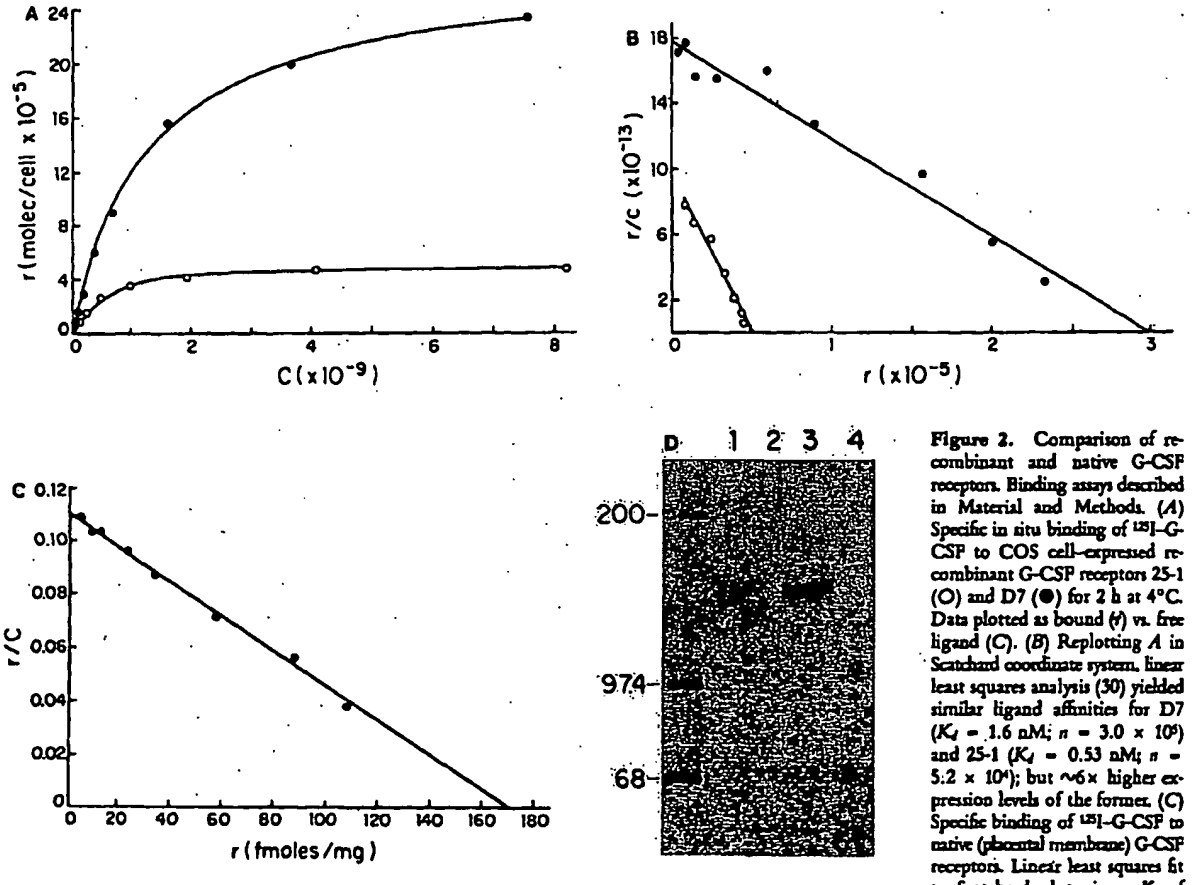

Figure 2. Comparison of recombinant and native G-CSF receptors. Binding assays described in Material and Methods. (*A*) Specific in situ binding of ^{125}I-G-CSF to COS cell-expressed recombinant G-CSF receptors 25-1 (○) and D7 (●) for 2 h at 4°C. Data plotted as bound (*r*) vs. free ligand (*C*). (*B*) Replotting *A* in Scatchard coordinate system, linear least squares analysis (30) yielded similar ligand affinities for D7 (K_d = 1.6 nM; n = 3.0 × 10^5) and 25-1 (K_d = 0.53 nM; n = 5.2 × 10^4); but ~6× higher expression levels of the former. (*C*) Specific binding of ^{125}I-G-CSF to native (placental membrane) G-CSF receptors. Linear least squares fit to Scatchard plot give a K_d of 0.70 nM, similar to both recombinant receptors. (*D*) Affinity cross-linking of native (placental; lanes *1* and *2*) and recombinant (COS-expressed; lanes *3* and *4*) in the absence (*1* and *3*) or presence (lanes *2* and *4*) of a 200-fold molar excess of unlabeled G-CSF. The calculated receptor M_r, after subtraction of ligand molecular weight, is ~150,000 in both cases. Cross-linking conditions described in Materials and Methods.

cDNA appears, therefore, to encode the strict human homologue of the murine receptor. At the protein level, these homologues are highly conserved in sequence (62% identity).

Comparison of Native and Recombinant G-CSF Receptors. The ligand-binding and affinity cross-linking characteristics of the recombinant G-CSF receptors encoded by D7 and 25-1, expressed in COS cells, are compared with those of native (placental) receptors in Fig. 2. All three receptors display a single class of binding sites, with equilibrium dissociation constants of 1.6 nM (D7), 0.53 nM (25-1), and 0.67 nM (placental). Expression levels of the D7 clone, however, were approximately sixfold higher than 25-1 (298,000 v. 52,000 sites/cell). Affinity cross-linking studies of COS-expressed D7 and placental G-CSF receptors detected a single subunit in each case with an apparent M_r of ~150,000. As the calculated protein molecular mass of the recombinant receptors are 92 kD (25-1) and 86 kD (D7), the G-CSF receptor is estimated to contain ~35% carbohydrate by weight. Thus both native and recombinant receptors share similar characteristics.

Expression of G-CSF Receptor mRNA. An antisense RNA transcript of the entire D7 sequence was used to probe Northern blots of total cellular RNAs isolated from a variety of sources (Fig. 3). A hybridizing band of ~3 kb was detected in placental RNA samples (lane *4*) and RNA isolated from human hematopoietic cells previously reported to express G-CSF receptors (17, 22, 23, 47), including the myelogenous leukemia cell line KG-1 (Fig. 3, lane 7), the promyelocytic cell line HL-60 (lane *1*), the premonocytic cell line U937 (lane *5*), bone marrow cells (lane 7), and peripheral blood granulocytes (lanes *2*, *3*), the latter containing particularly high levels, consistent with the prominent G-CSF responsiveness of this cell type. The KG-1 and peripheral blood granulocyte samples both show a minor additional hybridizing species at ~7 kb (lanes *2*, *3*, and 7), as did placental RNA upon longer exposure (data not shown). This species was not detectable in cytoplasmic placental RNA, suggesting it is a nuclear precursor (data not shown). The observed pattern of expression suggests that one or both of the G-CSF receptors cloned from the placental library also encode the receptors used by hematopoietic cells. Unexpectedly, we have also detected low levels of these transcripts in the HTLV-1–transformed T cell lines C-10 and MJ (lanes *6*, *10*) and the B lymphoblastoid cell lines RAJI and RPMI 1788 (lanes *9*, *11*), cells that are of lymphoid not myeloid lineage. The significance of this observation is unclear and requires further investigation. Under the stringent hybridization conditions used, no specific hybridization was seen with total RNAs isolated from HeLa cells (lane *8*), dermal fibroblasts, brain, or COS cells (data not shown).

Domain Structure and Sequence Homology of the G-CSF Receptor. A computer search of several databases queried with the entire G-CSF receptor sequence revealed significant homology of the G-CSF receptor extracellular region to three distinct groups of sequences: (*a*) members of the Ig superfamily (48), (*b*) the extracellular regions of all members of the recently identified hematopoietin (HP) receptor family

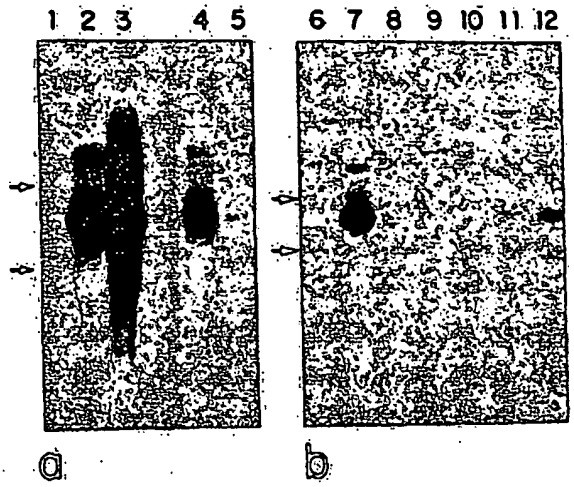

Figure 3. G-CSF receptor RNA analysis. Northern blots of human total cellular RNAs electrophoresed on formaldehyde agarose gels, hybridized with antisense D7 probe, and washed as described in Materials and Methods. The positions of the 18S and 28S ribosomal RNAs are indicated by arrows. All lanes contain 2.5 μg of total cellular RNA except lane *3*, which has 0.5 μg of total RNA to avoid overexposure. The blots were exposed at minus 80° for different times. (*A*) 2-h exposure of a blot of RNA samples from the following sources: HL-60 (lane *1*), peripheral blood granulocytes (lanes *2*, *3*), placenta (lane *4*), U937 (lane *5*). (*B*) 24-h exposure of a blot of RNAs isolated from the following sources: C-10 (lane *6*), KG-1 (lane 7), HeLA (lane *8*), RAJI (lane *9*), MJ (lane *10*), RPMI 1788 (lane *11*), aspirated pelvic bone marrow (lane *12*).

(44, 49–53) and (*c*) the type III homology units (FN3) of several vertebrate fibronectins (54) and neural cell adhesion molecules (NCAMs) (55, 56). Each of these homologies is localized to discrete regions of the extracellular portion of the G-CSF receptor. The NH_2-terminal 90 residues of the G-CSF receptor show statistically significant alignment scores (>3 SD) with several members of the Ig superfamily (data not shown). Fig. 4 *A* shows a consensus alignment of these NH_2-terminal residues with the NH_2-terminal sequences from the murine G-CSF receptor (25), human IL-6 receptor (57), and light chain domains of Ig NEW (58, 59). The G-CSF receptor contains an invariant Trp, two appropriately spaced Cys, and other residues in conserved positions that define Ig-like domains (48). Cys23 and Cys79 of the G-CSF receptor, therefore, are likely to form a disulfide loop characteristic of the Ig fold (60). This structural motif is further supported by the presence of sequences predicted to form the β strands characteristic of Ig domains (underlined Fig. 4 *A*).

The ~200 residue segment of the G-CSF receptor following the Ig-like moiety (Arg94 to Pro 299) shows strong homology to extracellular regions of all members of the HP receptor family (ALIGN scores Fig. 4 *D*) (44) and is shown aligned with the corresponding segments of the murine receptor and other selected family members in Fig. 4, *B* and *C*. This region contains the sequence features that define the

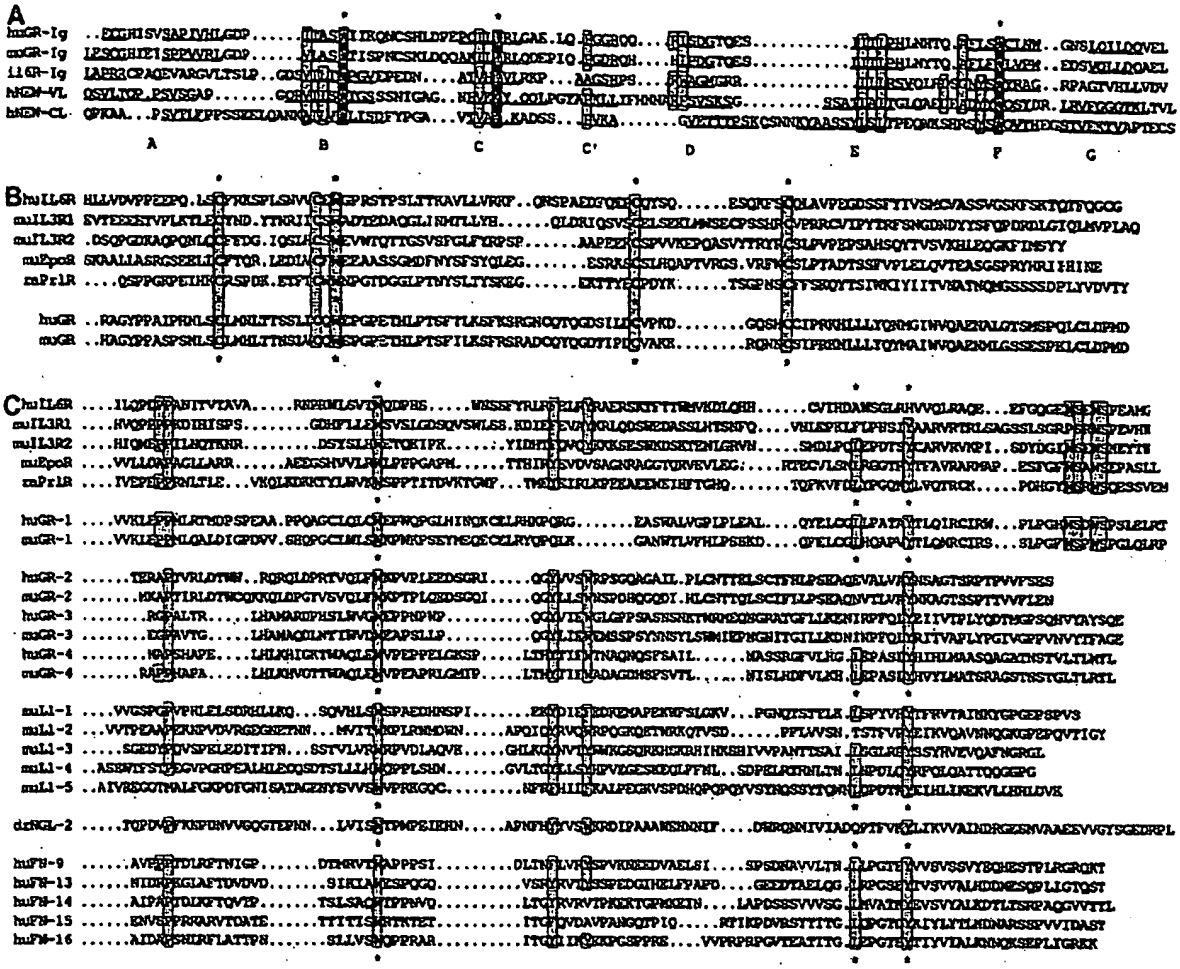

Figure 4. Homology domains of the G-CSF receptor. (*A*) Alignment of the human and murine G-CSF receptor NH_2-terminal Ig-like domain with the human IL-6 receptor Ig-like domain and the sequences from the light chains of IgNEW (58, 59). Boldface type indicates residues conserved in Ig domains. Underlined residues indicate the actual residues involved in the β-strands of the Ig domain folding pattern in the case of NEW and predicted residues comprising the same strands for the receptor sequences. (*B*) Alignment of the human and murine G-CSF receptor HP receptor family-defining region cysteine-rich domains (62) with sequences from the same region of the receptors for IL-6, IL-3, erythropoietin, and prolactin (53, 57, 77, 78). The conserved Cys and Trp residues of the HP receptor family (44, 49–53) are indicated by asterisks and shading. Cys residues are in boldface type. (*C*) Alignment of the four fibronectin type III (FN3) domains of the human and murine G-CSF receptor extracellular region with the FN3-like domains of the HP receptor family defining regions (62) of the receptors for IL-6, IL-3, erythropoietin, and prolactin and with the five FN3 repeats of murine L1 NCAM (55) the second FN3 repeat of Drosophila neuroglian (63) and four type III repeats of human fibronectin (64). Asterisks and shading indicate the three residues conserved in all 16 repeats of human fibronectin. Shading indicates other residues conserved in some FN3 domains and the WSXWS motifs. Cys and Trp residues are in boldface type. (*D*) Scores generated by the NBRF ALIGN program (38) for the indicated sequences.

D

Align Scores

Sequence	huGCSFR	huIL7R	muIL7R	huIL4R	muIL4R	huIL2Rβ	huIL6R	huLSR1	huLSR2	GMCFR	EPOR	PRLR	PRLR	GHBR	GHBR
huGCSFR	-	5.8	3.8	4.9	5.3	5.5	5.6	5.7	6.2	6.0	6.2	10.7	11.4	5.6	6.3
huIL-7R	25.1	-	33.8	5.8	5.9	3.8	4.8	3.1	6.7	2.4	5.6	6.0	6.2	4.3	3.9
muIL-7R	22.9	67.2	-	5.6	4.1	4.8	4.6	4.0	5.7	3.1	6.9	5.5	7.1	3.6	4.5
huIL-4R	21.6	17.2	19.3	-	26.9	5.4	8.2	4.8	8.1	5.7	8.4	3.8	5.7	3.1	3.3
muIL-4R	20.7	17.0	19.1	50.8	-	4.7	6.1	4.9	7.3	4.8	5.7	4.6	6.5	1.7	1.8
huIL-2Rβ	22.0	18.7	21.1	18.7	21.2	-	5.4	3.4	8.0	7.3	7.3	4.8	4.3	4.4	2.6
huIL-6R	18.4	19.5	19.1	23.3	21.7	21.5	-	4.5	5.8	6.5	7.0	8.7	9.3	5.5	6.5
muIL-3R1	21.1	20.1	19.3	22.7	22.7	18.3	22.0	-	7.6	5.7	4.3	6.2	6.4	5.3	5.0
muIL-3R2	20.4	20.1	22.0	24.6	24.3	23.5	23.1	21.9	-	8.4	9.9	7.6	6.2	5.2	4.5
huGMCSFR	17.3	17.9	18.2	18.4	18.6	18.5	17.0	18.9	20.3	-	9.7	7.1	6.1	5.9	5.3
muEPOR	18.6	18.7	20.9	20.5	17.1	19.9	20.4	13.2	22.0	19.6	-	9.4	9.9	6.2	6.6
rnPRLR	28.2	19.6	20.6	17.5	21.8	19.0	23.7	19.3	19.5	21.9	21.6	-	47.0	13.3	13.3
rbPRLR	27.4	25.5	20.6	21.1	20.8	21.9	25.8	18.3	16.5	20.9	23.7	76.9	-	14.4	14.5
huGHBR	18.1	22.4	18.7	20.3	20.0	19.5	20.2	22.4	23.8	18.1	18.0	33.3	35.4	-	51.0
rbGHBR	20.6	22.6	20.6	19.8	20.3	18.0	21.2	21.5	20.7	17.9	24.2	34.4	35.4	68.3	-

Percent Identity

HP receptor family; notably a Trp and four Cys residues (*boxes*, *asterisks*: Fig. 4 *B*) conforming to a conserved pattern (49–53) found in nearly all HP receptors (44) and a COOH-terminal WSXWS motif (50–53) which has proven to be a hallmark of the HP receptor family (44). The four Cys residues form two successive disulfide loops in the growth hormone receptor (61), and it is likely that a similar pairing occurs in the G-CSF receptor between Cys107 and 118, and Cys153 and 162. A recent sequence analysis of the HP receptor family-defining region (62) has suggested it can be resolved into two distinct elements each ~100 residues in length: one NH_2-terminal "cysteine-rich" region and a COOH-terminal "cysteine-poor" region, the latter homologous to fibronectin type III repeats. Although in the G-CSF receptor these two regions do not differ significantly in number of Cys residues (NH_2-terminal; 6; COOH-terminal, 5), the last 100 residues of the region do contain the sequence features characteristic of FN3 repeats, as shown by alignment with examples of FN3 repeats from human fibronectin (54) and two neural cell adhesion molecules, murine L1 (55) and Drosophila neuroglian (63) in Fig. 4 C. The FN3-like character of the region is demonstrated by the presence of three residues, Trp229, Leu274, and Tyr279 (asterisks), in the pattern which is the sole sequence feature absolutely conserved in all of the type III repeats of fibronectin (54, 64). Although this region of the G-CSF receptor exceeds the low Trp and Cys content characteristic of FN3 repeats (54) its designation as an FN3-like domain is further supported by statistically significant ALIGN scores when compared with 14 of the 16 type III repeats of human fibronectin (data not shown).

The ~300 residues of the G-CSF receptor bordered by the WSXWS motif and the transmembrane region also show significant homology to FN3 repeats of several vertebrate fibronectins and NCAMs, suggesting this region consists of three additional repeats of this element. These proposed FN3-like domains are shown aligned with the corresponding segments of the murine receptor and the examples of FN3 repeats in Fig. 4 C. These three FN3-like domains of the G-CSF receptor contain the conserved Trp and Tyr residues, described above, but only the last domain contains the hallmark Leu, a residue only partially conserved in NCAM FN3 repeats. These domains of the G-CSF receptor also contain a pair of aromatic residues common to the FN3-like domains of many NCAMs and fibronectins (*boxed*). The four proposed FN3-like domains of the G-CSF receptor each contain at least three Trp residues, and in this respect resemble the FN3 repeats of NCAMs rather than those of fibronectin. The proposed second, third, and fourth FN3 domains of the G-CSF receptor gave significant ALIGN scores respectively to 4, 13, and 15, of the 16 type III repeats of human fibronectin (data not shown).

Discussion

Here we report the isolation and characterization of two distinct types of G-CSF receptor cDNAs from a human placental library. The equilibrium ligand binding and cross-linking characteristics of the recombinant receptors are similar to those of native receptors on placental membranes (Fig. 2). The D7 and 25-1 cDNAs are predicted to encode integral membrane glycoproteins, 759 and 812 amino acids in mature length, respectively. The predicted molecular masses of these receptors, 86 and 92 kD, are substantially less than the ~150 kD inferred by affinity crosslinking, suggesting that some or all of the nine potential N-linked glycosylation sites contain carbohydrate. The two receptors share identical extracellular (603 aa) and transmembrane (26 aa) regions, as well as the first 96 residues in their cytoplasmic regions, but have alternate COOH-terminal sequences of 34 residues (D7) and 87 residues (25-1). The nucleotide sequences of the two cDNAs indicate that they are probably derived from alternatively processed transcripts of the same gene, since they differ only with respect to a 419-bp insert in the 25-1 cDNA that appears to be an unspliced intron. A recently reported murine G-CSF receptor cDNA (25) encodes a protein that shows strong homology to both placental G-CSF receptors up to the splice point in the cytoplasmic domain, after which the homology continues only in the 25-1 clone, indicating it is the strict human homologue of the reported murine receptor. It is unclear if the reported murine G-CSF receptor cDNA sequence has the splicing potential to encode an alternate COOH-terminus. Inspection of the murine sequence reveals a very similar (murine, CAG GTCCTC; human, CAG GTCCTT) potential splice donor sequence located within the same DQ/VLY peptide sequence as the human 25-1 cDNA. However, while there are potential splice acceptor sites in the murine sequence, translation of the sequences downstream in all three reading frames reveal no significant homology to the D7 type COOH terminus. Thus, generation of a D7-type cytoplasmic terminus in murine G-CSF receptors might be possible if an alternative splice acceptor site exists in 3′ sequences of the murine gene.

Differential splicing results in the tissue-specific expression of transcripts encoding alternate cytoplasmic domains for at least two other cell surface proteins, rat liver prolactin (PRL) receptor (65) and chicken NCAM (66). The tissue specificity of expression of the potential G-CSF receptor isoforms remains to be determined at both the mRNA and protein level, but a preliminary analysis with specific oligonucleotide probes suggests human granulocytes express predominantly transcripts of the 25-1 type (data not shown). This raises the possibility that the D7 receptor is specifically expressed at higher levels in nonhematopoietic cells such as placenta, and suggests that the alternate cytoplasmic domains may confer functional differences to the two receptors. It has been proposed that the smaller form of the PRL receptor functions in ligand transport across epithelial barriers in liver rather than in signal transduction (65). By analogy the D7 isoform of the G-CSF receptor may serve to transport or sequester G-CSF in placental tissues. Alternatively, the two isoforms may differ in signal transduction properties, reflecting in part, the diverse biological effects of G-CSF. While the signal transduction mechanism of the G-CSF receptor is unclear, it is interesting that the 25-1 receptor, unlike D7, does contain one potential C kinase phosphorylation site (46). Signal transduction may also be effected through a distinct subunit with which the ligand-

G-CSF receptor complex interacts, as is found in the gp130–IL-6 receptor system (67). Conceivably, this subunit may be gp130 itself, and it is interesting, in this regard, that IL-6 and G-CSF show significant sequence homology (68), and their receptors share a similar domain composition (see below). Both G-CSF receptor cytoplasmic domains contain a high proportion of Pro and Ser residues, like those of many other HP receptor family members (44), the significance of which remains to be elucidated. The cytoplasmic sequence of these receptors may influence stability, cellular localization, or association with other membrane proteins.

The extracellular region of the G-CSF receptor consists of three distinct regions of homology to other cell surface proteins: (*a*) a ~90 residue NH_2-terminal Ig-like region, (*b*) a ~200 residue HP receptor superfamily-defining region, and (*c*) ~300 residues of three tandem FN3-like repeats. Consistent with the proposal of Patthy (62), the COOH-terminal 100 residue segment of the HP receptor family-defining region of the G-CSF receptor appears to be an FN3-like domain, albeit one with an elevated Trp and Cys content that is unique among FN3 repeats. Since many protein domains are ~100 residues in length, it is likely that the NH_2-terminal "cysteine-rich" or double-loop region of the HP receptor-defining region is, like the WSXWS-containing FN3 element, a discrete structural domain. Thus, the structure of the G-CSF receptor extracellular region can be resolved into the 6 domains of ~100 residues each shown schematically in Fig. 5. By this analysis, the G-CSF receptor appears to be a mosaic of four types of domains, two found associated only in HP receptors (double-loop and WSXWS-FN3) and two found together in NCAMs (NH_2-terminal Ig and membrane-proximal FN3s). Given the likelihood that the Ig superfamily molecules of the immune system arose from NCAM-like ancestors (48), it is tempting to speculate that the G-CSF receptor retains the domain structure of an early intermediate in the evolution of the HP receptor superfamily from these same ancestors. Successive deletion of the terminal extracellular domains of a G-CSF receptor-like molecule could thus yield all known HP receptor domain structures; deletion of the three FN3 domains yields an IL-6 receptor-like structure, and further deletion of the Ig domain leads to the core domain structure of most HP receptors, which is duplicated in the case of the IL-3 receptor. Key to such a proposal is determination of the origin of the cysteine-rich or double-loop domain, thus far a unique domain feature found only in the HP receptors, but which, like the Ig and FN3 domains, may also have arisen from a domain in some NCAM-like ancestor.

The function of these structural domains in the G-CSF receptor is unclear. The HP receptor family-defining region, which comprises the entire extracellular region of many of these receptors, presumably contains the ligand binding site of the G-CSF receptor. The Ig and extra FN3-like domains of the G-CSF receptor may confer additional activities to this receptor, similar to the complex functions recognized for these domains in other cell surface molecules. These generally appear to involve participation in some form of cell recognition or adhesion. Both types of domains are capable of homotypic and heterotypic interactions (48, 69) that might lead to receptor self-association or binding to other proteins. Specific functions have been attributed to some type III repeats of fibronectin, including the binding of cells and heparin (70, 71) and heparin binding activity is also a property of at least one neural cell adhesion molecule (72). The potential for simultaneous recognition of heparin and G-CSF by the G-CSF receptor would have interesting functional implications, especially in light of the affinity of the heparin component of extracellular matrix for CSFs (73, 74). The particular response of a cell to G-CSF could thus depend on its adherence or that of G-CSF to the extracellular matrix. Alternatively it could allow G-CSF to mediate or specify interactions between cells and matrix, thus, directing margination or chemotaxis. The cloning of the human G-CSF receptor will provide reagents useful in the further elucidation of the biological roles of G-CSF and may allow development of new diagnostic or therapeutic agents.

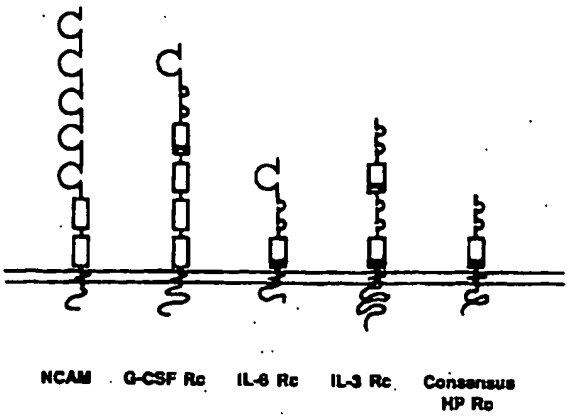

Figure 5. Schematic representation of the nominal structure of hematopoietin receptors and a typical neural cell adhesion molecule. Ig-like domains are shown as large loops. FN3 domains are shown as rectangular boxes. The cysteine-rich, double-loop domains of HP receptor family-defining regions are shown as a pair of small loops, to represent the disulfide pairing determined for human growth hormone receptor (61). The FN-3 like domain of HP receptor family-defining regions is designated as a rectangular box crossed by a heavy bar representing the WSXWS motif. Sequences represented are rat NCAM (reference 79 as drawn in 56), IL-6 receptor (57), and IL-3 receptor (53).

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Functional domains of the granulocyte colony-stimulating factor receptor

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The granulocyte colony-stimulating factor (G-CSF) receptor has a composite structure consisting of an immunoglobulin(Ig)-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains in the extracellular region. Introduction of G-CSF receptor cDNA into IL-3-dependent murine myeloid cell line FDC-P1 and pro-B cell line BAF-B03, which normally do not respond to G-CSF, enabled them to proliferate in response to G-CSF. On the other hand, expression of the G-CSF receptor cDNA in the IL-2-dependent T cell line CTLL-2 did not enable it to grow in response to G-CSF, although G-CSF could transiently stimulate DNA synthesis. Mutational analyses of the G-CSF receptor in FDC-P1 cells indicated that the N-terminal half of the CRH domain was essential for the recognition of G-CSF, but the Ig-like, FNIII and cytoplasmic domains were not. The CRH domain and a portion of the cytoplasmic domain of about 100 amino acids in length were indispensable for transduction of the G-CSF-triggered growth signal.

Key words: cytokine receptor family/granulocyte colony-stimulating factor/hemopoietic cells/signal transduction

Introduction

The proliferation and differentiation of hemopoietic cells such as granulocytes, macrophages, T cells and B cells are regulated by a family of cytokines including colony-stimulating factors (CSFs) and interleukins (ILs) (reviewed in Metcalf, 1989). Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein of 174 (human G-CSF) or 178 (murine G-CSF) amino acids, and specifically stimulates colony formation of neutrophilic granulocytes from bone marrow cells (Nagata *et al.*, 1986a,b; Tsuchiya *et al.*, 1986). G-CSF also stimulates proliferation of murine and human myeloid leukemia cells such as NFS-60 (Weinstein *et al.*, 1986), AML-193 (Santoli *et al.*, 1987) and OCI/AML 1 (Nara *et al.*, 1990) cells, while murine WEHI-3B D^+ and 32DC13 cells can be induced by G-CSF to differentiate into granulocytes or monocytes (reviewed in Nicola, 1989; Nagata, 1990). G-CSF produced by stroma cells in bone marrow seems to play an essential role in maintaining the number of neutrophilic granulocytes in peripheral blood. G-CSF is produced also by macrophages stimulated with endotoxins (Metcalf and Nicola, 1985; Nishizawa and Nagata, 1990), and by fibroblasts or endothelial cells treated with TNF-α or IL-1 (Seelentag *et al.*, 1987; Koeffler *et al.*, 1988). The G-CSF thus accumulated in blood appears to be responsible for granulocytosis during the inflammatory process. Since administration of G-CSF to animals caused a marked granulopoiesis (Tsuchiya *et al.*, 1987), the substance is currently under clinical trials for patients suffering from granulopenia (Morstyn *et al.*, 1989).

Despite the biological importance of G-CSF, its mechanism of signal transduction has not been elucidated. A single class of high affinity receptor for G-CSF [dissociation constant (K_d) = 100-500 pM] is present on the precursor and mature cells of neutrophilic granulocytes as well as myeloid leukemia cells (Nicola and Peterson, 1986; Park *et al.*, 1989; Fukunaga *et al.*, 1990b). Purification of the G-CSF receptor from mouse myeloid leukemia NFS-60 cells has indicated that the receptor has an M_r of 100 000-130 000, and the monomeric form of this protein binds G-CSF with a low affinity (K_d = 2.6-4.2 nM), while its oligomeric forms show high affinity binding to G-CSF (K_d = 120-360 pM) (Fukunaga *et al.*, 1990b). Recently, we have isolated the cDNAs for murine (Fukunaga *et al.*, 1990a) and human G-CSF receptors (Fukunaga *et al.*, 1990c); expression of these receptor cDNAs in monkey COS cells gave rise to proteins which specifically bound G-CSF with a high affinity, suggesting that the single polypeptide encoded by the cDNA is sufficient to constitute the high affinity binding site for G-CSF.

Murine and human G-CSF receptors consist of 812 and 813 amino acids, respectively, and contain a single transmembrane domain (Fukunaga *et al.*, 1990a,c). In agreement with the fact that G-CSF has no species specificity between human and mouse, the amino acid sequences of human and murine G-CSF receptors have a considerable similarity (62.5% identity) (Fukunaga *et al.*, 1990c). In the extracellular domain (~600 amino acids) of the G-CSF receptor, there is a region of ~200 amino acids which shows significant homology to the receptors for IL-3-7, erythropoietin and GM-CSF, and the β-chain for the IL-2 receptor (Bazan, 1990a). In the case of the G-CSF receptor, this region (referred to as the cytokine receptor-homologous domain or CRH domain) is followed by a domain (~300 amino acids) consisting of three fibronectin type III (FNIII) modules, which is homologous to chicken contactin (Fukunaga *et al.*, 1990a). The cytoplasmic region of the G-CSF receptor has a limited similarity to that of the IL-4 receptor (Mosley *et al.*, 1989), and like other members of the cytokine receptor family, the region does not appear to contain the domain with kinase or other enzymatic activity. Very recently, the human cDNA for the IL-6 signal transducer, gp130, was isolated (Hibi *et al.*, 1990). The overall structure of gp130 is remarkably similar to that of the G-CSF receptor, and the similarity of human G-CSF receptor and gp130 is 46.3% on the amino acid sequence level, when conservative substitutions are included.

In order to investigate the role of the G-CSF receptor in the G-CSF-dependent signal transduction, we have expressed

the G-CSF receptor cDNA in various hematopoietic cell lines using a promoter of human elongation factor 1α gene. The G-CSF receptor could function as a transducer for the G-CSF-triggered growth signal in IL-3-dependent FDC-P1 and BAF-B03 cells, but not in IL-2-dependent CTLL-2 cells. Utilizing this expression system, we have identified the functional domains of the G-CSF receptor.

Results

Establishment of cell lines expressing the G-CSF receptor

Mouse myeloid precursor cell line FDC-P1 and pro-B cell line BAF-B03 require IL-3 for their growth, whereas the growth of murine cytotoxic T cell line CTLL-2 is strictly dependent on IL-2. In order to examine whether the cloned G-CSF receptor can transduce the G-CSF-triggered signal in these cells, we have established transformed cell lines expressing the G-CSF receptor cDNA. For this purpose, we took advantage of a recently constructed mammalian expression vector, pEF-BOS, which utilizes a constitutive promoter of the human polypeptide chain elongation factor 1α (EF-1α) gene (Uetsuki *et al.*, 1989), and which works very efficiently in various cell lines (Mizushima and Nagata, 1990). The pEF-BOS plasmid harboring a murine G-CSF receptor cDNA (pI62) (Fukunaga *et al.*, 1990a) or human G-CSF receptor cDNA (pHQ3) (Fukunaga *et al.*, 1990c) was transfected into FDC-P1, BAF-B03 and CTLL-2 cells,

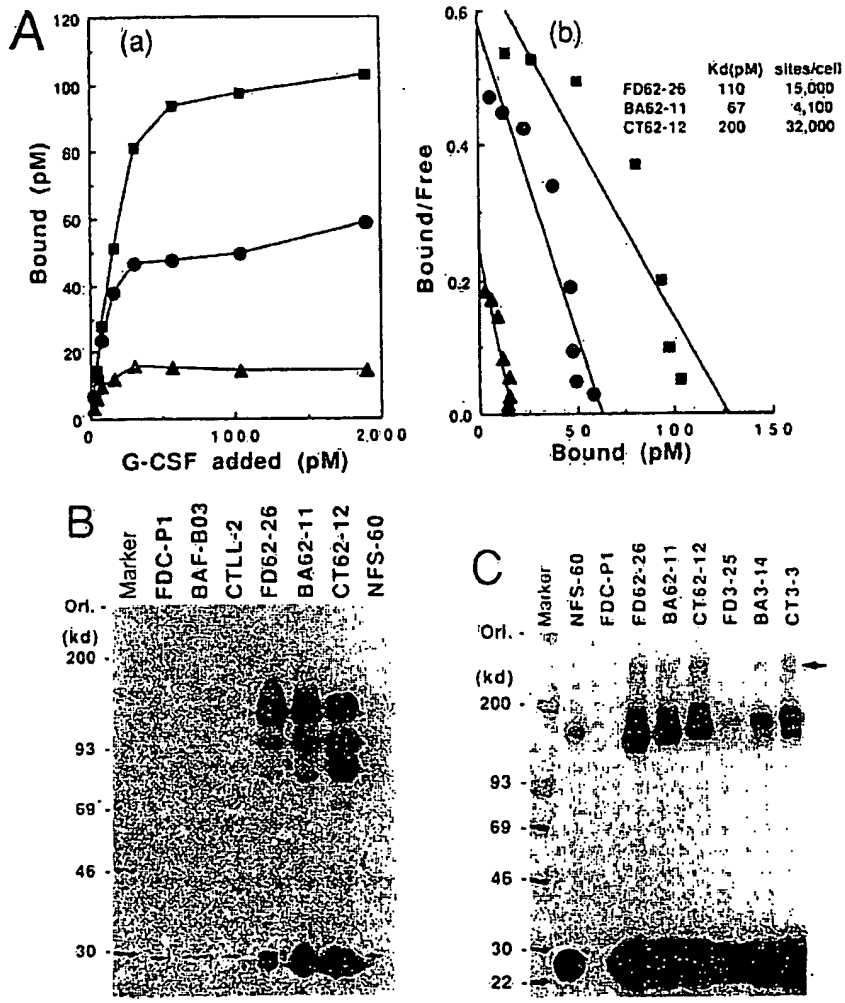


Fig. 1. Expression of mouse and human G-CSF receptor in hemopoietic cell lines. (A) G-CSF binding characteristics in transformant clones. (a) Saturation binding of [^{125}I]G-CSF to transformant clones expressing mouse G-CSF receptor cDNA. The transformants derived from FDC-P1, BAF-B03 and CTLL-2 were designated FD62, BA62 and CT62, respectively. 2.5×10^6 cells/ml were incubated for 3 h at 4°C with various amounts of [^{125}I]G-CSF. The specific binding of [^{125}I]G-CSF to FD62-26 (●), BA62-11 (▲) or CT62-12 (■) was determined as the difference between total binding and non-specific binding which was measured in the presence of 500 nM unlabeled G-CSF. (b) Scatchard plot of G-CSF binding data shown in (a). (B) Immunoblotting of mouse G-CSF receptor with anti-MR9 serum. Cell lysates prepared from parental cell lines, their transformant clones and NFS-60 were analyzed by immunoblotting with an anti-mouse G-CSF receptor serum (anti-MR9) as described in Materials and methods. As size markers, ^{14}C-labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel ('Marker' lane). The positions of the mature and less glycosylated G-CSF receptors are indicated by solid and open arrowheads, respectively. (C) Chemical cross-linking of the mouse and human G-CSF receptors with radioiodinated G-CSF. NFS-60, FDC-P1 and transformed cell clones (2×10^7 cells/ml) expressing mouse (FD62-26, BA62-11 and CT62-12) or human (FD3-25, BA3-14 and CT3-3) G-CSF receptor cDNA were incubated for 3 h at 4°C with 500 pM [^{125}I]G-CSF and chemically cross-linked with disuccinimidyl suberate and disuccinimidyl tartarate as previously described (Fukunaga *et al.*, 1990b). The cell lysate (50 μg protein) was analyzed by SDS–PAGE, and the gel was dried and exposed to X-ray film.

together with a plasmid carrying the neomycin resistance gene. G418-resistant transformants were tested for the ability to bind [^{125}I]G-CSF, and several independent clones were isolated after limiting dilution from the binding-positive transformants.

The properties of the G-CSF binding to these stable transformants were first examined. The parental FDC-P1, BAF-B03 and CTLL-2 cells did not bind G-CSF (data not shown). On the other hand, the cell clones of FD62, BA62 and CT62, transformed with the mouse G-CSF receptor cDNA, bound G-CSF with high affinities (K_d = 110, 70 and 200 pM, respectively) (Figure 1A). The numbers of the G-CSF receptor expressed in clones of FD62 and BA62 were constantly 4000–15 000 sites per cell, while clones of C-T62 expressed the mouse G-CSF receptor in the range 20 000–30 000 sites per cell. The transformants with the human G-CSF receptor cDNA (FD3, BA3 and CT3 cells) also expressed only a high affinity G-CSF receptor (K_d = 170–300 pM) at 2400–20 000 sites per cell (data not shown). These K_d values are consistent with our previous results obtained with COS cells (Fukunaga *et al.*, 1990a,c) and confirm that the single polypeptide coded by the cloned G-CSF receptor cDNA is sufficient to constitute a high affinity binding site for G-CSF.

For the immunological detection of the G-CSF receptor, two proteins containing a portion of the extracellular domain (MR1, amino acids 36–326) or cytoplasmic domain (MR9, amino acids 631–812) of the mouse G-CSF receptor were produced in *Escherichia coli*, and polyclonal antibodies against these proteins were prepared in rabbits. An immunoblot using anti-MR9 antiserum identified three molecular species varying in apparent molecular size (125–135 kDa, 105–110 kDa and 85–90 kDa) in the transformant clones as well as in mouse NFS-60 cells which express the endogenous G-CSF receptor (Figure 1B). The bands detected at ~30 kDa in the transformant clones are probably degraded forms of the receptor. The 125–135 kDa species seems to be the mature, cell surface receptor because the cross-linking of the cell surface receptor with radioactive G-CSF gave only a ligand–receptor complex with an apparent M_r of 150–160 kDa (Figure 1C). The other two species are likely to be less glycosylated or non-glycosylated intermediates. The small difference in molecular sizes of the receptor (Figure 1B and C) among the cell types could be due to difference in their glycosylation. Cross-linking experiments also confirmed that the transformant clones FD3-25, BA3-14 and CT3-3 expressed human G-CSF receptor (Figure 1C). We have often observed an additional cross-linked complex with higher molecular size in cells expressing a large number of G-CSF receptors (Figure 1C, indicated by an arrow). Since this complex has an M_r of ~300 kDa, it may represent the cross-linked dimer of the G-CSF receptor with which [^{125}I]G-CSF was cross-linked. The immunoblot analysis sometimes showed a faint band at 125–135 kDa in the parental FDC-P1, BAF-B03 and C-TLL-2 cells (Figure 1B). This band seems to be a non-

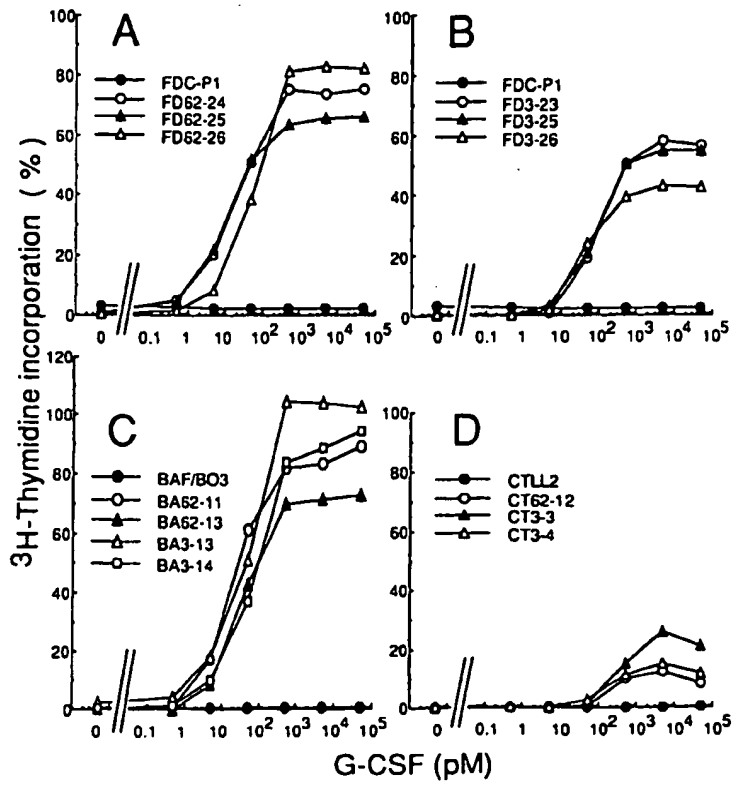


Fig. 2. G-CSF-dependent DNA synthesis of transformants expressing mouse or human G-CSF receptor cDNA. Parental cells and their transformed cell clones expressing mouse or human G-CSF receptor were cultured with 0–50 nM mouse G-CSF, and incorporation of [^{3}H]thymidine into cells was measured. The results are presented as the percentage of the maximum [^{3}H]thymidine incorporation observed with IL-3 (A, B and C) or IL-2 (D) in the respective cells. (A and B) FDC-P1 and its transformant clones expressing the mouse (A) or human (B) G-CSF receptor cDNA. (C) BAF-B03 and its transformant clones expressing mouse or human G-CSF receptor. (D) CTLL-2 and its transformant clones expressing mouse and human G-CSF receptor.

specific one because these cells neither bound G-CSF nor gave the cross-linked complex with [^{125}I]G-CSF (Figure 1C, data not shown).

G-CSF receptor can transduce the growth signal in FDC-P1 and BAF-B03 cells but not in CTLL-2 cells

The effect of G-CSF on the growth properties of the transformants expressing the G-CSF receptor was then examined by [^{3}H]thymidine uptake assay. As shown in Figure 2, none of the parental cell lines responded to G-CSF, whereas transformants derived from FDC-P1 and BAF-B03 responded to G-CSF in a concentration-dependent manner. In the FDC-P1 transformants, clones expressing murine G-CSF receptor (clones of FD62) responded slightly more efficiently than the clones expressing human G-CSF receptor (clones of FD3). The maximum [^{3}H]thymidine uptakes of FD62 and FD3 obtained at >500 pM G-CSF were 70–80% and 50–60%, respectively, of that observed with an excess of murine IL-3 (Figure 2A and B). In BAF-B03 transformants, human and murine G-CSF receptors were equally effective, and the maximum response obtained at 500 pM of G-CSF was almost comparable to that observed with mouse recombinant IL-3 (Figure 2C). In accordance with the high responsiveness of these transformants to G-CSF, 500 pM G-CSF could support the long-term growth of clones of FD62, FD3, BA62 and BA3 in the absence of IL-3 (Figure 3). On the other hand, G-CSF stimulated very weakly the DNA synthesis of the CTLL-2 transformants (clones of CT62 and CT3) (Figure 2D). The response was <30% of that observed with an excess of IL-2, and this weak responsiveness was insufficient to support the long-term proliferation or survival of these cells in the absence of IL-2 (Figure 3).

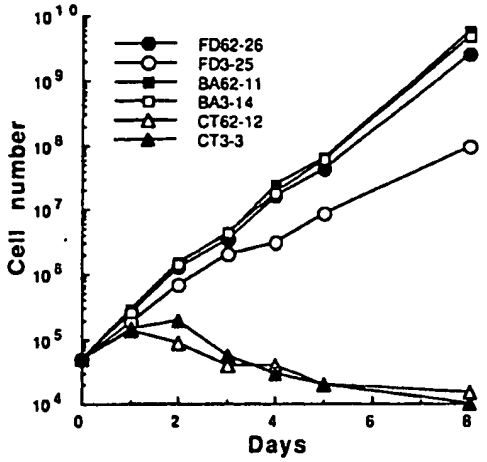
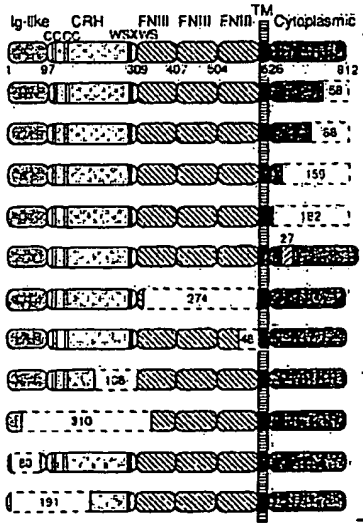


Fig. 3. G-CSF-dependent long-term growth of transformants expressing G-CSF receptor cDNA. Transformants growing in IL-3 or IL-2 were washed thoroughly and cultured at 5×10^4 cells/ml in RPMI1640 containing 10% FCS and 500 pM mouse G-CSF. Cell numbers were counted daily and cell concentrations were kept less than 10^6 cells/ml by appropriate dilution with the same medium.

Construction and expression of cDNAs carrying various mutations in the subdomains of the G-CSF receptor

As shown in Figure 4, the G-CSF receptor contains a single transmembrane region which divides the molecule into two regions, the extracellular and the cytoplasmic. The extracellular region can be further divided into five subdomains, an immunoglobulin(Ig)-like domain, a CRH domain and three FNIII domains, based on homology with other proteins (Fukunaga *et al.*, 1990a; Bazan 1990a) and the exon–intron organization of the G-CSF receptor gene (Y.Seto, R.Fukunaga and S.Nagata, unpublished results).



Mutant	G-CSF binding Kd (pM)	G-CSF binding sites/cell	G-CSF-dependent growth
Wild type	203 ± 87	5500 ~15000	+++
A Δ(755-812)	232 ± 28	9300 ~13000	+++
E Δ(725-812)	178 ± 19	4800 ~ 6700	++
P Δ(654-812)	207 ± 33	3200 ~ 7400	-
X Δ(631-812)	174 ± 43	1500 ~ 6200	-
11 Ins27(856)	182 ± 39	3300 ~11000	+
C Δ(324-597)	475 ± 71	1100 ~ 2300	+
7 Δ(553-598)	175 ± 20	350 ~ 820	+
17 Δ(201-306)	11000 ±1600	8300 ~ 8400	-
W Δ(37-346)	-	no binding	-
G Δ(5-64)	3650 ± 180	2100 ~ 4100	+
Y Δ(5-193)	-	no binding	-

Fig. 4. Summary of mutational analysis of the G-CSF receptor. The extracellular domain of the G-CSF receptor is putatively divided into five subdomains, an Ig-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains (top). Four cysteine residues and a WSXWS motif conserved in the CRH domain are also indicated. The deletion mutants of mouse G-CSF receptor (mutants A, E, P, X, C, W, G and Y) and three variants (variants 11, 7 and 17), are shown schematically below the wild-type receptor. The portions deleted in the mutants are represented by the dotted lines and the number of amino acids deleted in each mutant is indicated. In all wild-type and mutant cDNAs, the coding region for the mature protein is preceded by an N-terminal signal sequence, which is not shown in the figure. The table at the right shows the G-CSF binding characteristics of the mutated receptor and their abilities to transduce the G-CSF-triggered growth signal.

In order to assign the functional domains of the G-CSF receptor, we have constructed a series of murine G-CSF receptor cDNAs which have various deletions in these subdomains. In the first set of deletion mutants (mutants A, E, P and X), the cytoplasmic region of the receptor was progressively deleted from the C-terminal end up to amino acid positions 754, 724, 653 and 630, respectively (Figure 4). For the extracellular region, four deletion mutants (C, W, G and Y) were constructed. In addition to these constructions, we have used as mutants variant receptors whose cDNAs were isolated from human and mouse cDNA libraries. The human G-CSF receptor variant (mutant 11) contains a 27 amino acid insertion between amino acid positions 657 and 658 in the cytoplasmic domain (Fukunaga *et al.*, 1990c), while murine variants carry the deletion of the C-terminal half of the third FNIII domain (mutant 7) or the C-terminal half of the CRH domain (mutant 17) (Figure 4). These G-CSF receptor variants seem to be generated by alternative splicing of the precursor RNA (Y.Itoh, E.Ishizaka-Ikeda, Y.Seto, R.Fukunaga and S.Nagata, unpublished results).

FDC-P1 cells were transformed with expression plasmids carrying the mutated cDNA as described above. Stable transformants expressing each mutant were isolated and designated FD-A, FD-E, FD-P and so forth. Expression of the mutated receptor in these transformants was first analyzed by immunoblot analysis using anti-MR9 antiserum. As shown in Figure 5A, the mature and less glycosylated receptor with molecular weights expected from the size of the deletion was observed in all transformant clones except for clones of FD-P and FD-X, which were transformed with mutants containing little or no part of the MR9 polypeptide. A relatively weak intensity observed in mutants A and E was also due to deletion of parts of the cytoplasmic domain. An immunoblot with anti-MR1 antiserum recognizing a part of the extracellular domain of the mouse G-CSF receptor showed bands in transformant clones for all mutants except for the mutant W which had a deletion of the region recognized by anti-MR1 serum (Figure 5B). Furthermore, as shown in Figure 5C, cross-linking with [^{125}I]G-CSF gave a complex of the expected size in all transformant clones which bind G-CSF with a high affinity (Figure 4). In contrast, no cross-linked complex was observed in clones FD-17 (Figure 5C, lane 6), FD-G, FD-W and FD-Y (data not shown) which have a low or undetectable affinity to G-CSF (Figure 4). Expression of a variant of the human G-CSF receptor in FD11-10 cells was confirmed by the cross-linking experiment (Figure 5C). These results indicate that all mutants of the G-CSF receptor were expressed in the transformants, although the expression levels of mutants C, 7 and G, which have deletions in the FNIII or Ig-like domain, were relatively low in all clones so far tested (Figures 4 and 5).

The CRH domain is responsible for binding of G-CSF

Using the FDC-P1 transformants expressing the mutated G-CSF receptor, we have located the region of the receptor essential for binding of the ligand. Typical Scatchard plots of the G-CSF binding data with these transformants are shown in Figure 6, and summarized in Figure 4. The mutant

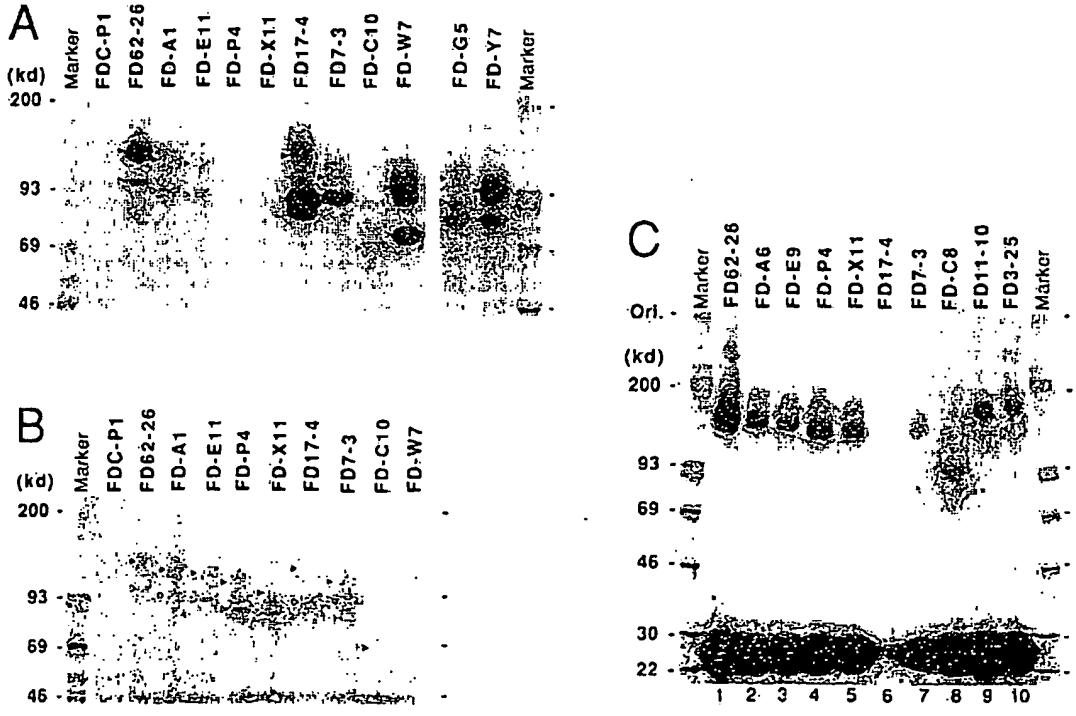


Fig. 5. Expression of G-CSF receptor mutants in FDC-P1 transformants. (A and B) Cell lysates of FDC-P1-derived transformants expressing wild-type or mutant cDNA of mouse G-CSF receptor were analyzed by immunoblotting with the anti-MR9 antiserum (A), or anti-MR1 antiserum (B) as described in Figure 1B. (C) The wild-type and mutant G-CSF receptors expressed in FDC-P1 transformants were cross-linked with [^{125}I]G-CSF and analyzed as described in Figure 1C. The cell lysate of 50 μg (lanes 1–5), 200 μg (lanes 6–8) or 100 μg (lanes 9 and 10) protein was electrophoresed.

X, containing only five amino acids in the cytoplasmic region, as well as mutants A, E, P and 11, could bind G-CSF with the same affinity as the wild-type receptor (K_d ~ 200 pM). These results indicate that the truncations or mutations in the cytoplasmic region do not affect the extracellular binding of G-CSF to the receptor.

Two mutants containing deletions in the FNIII domains (mutants 7 and C) could bind G-CSF with high affinities, although mutant C showed slightly lower affinity (K_d = 480 pM) than the wild-type receptor. On the other hand, mutant 17, which lacks the C-terminal 108 amino acids of the CRH domain, and mutant G, which lacks the Ig-like domain, bound G-CSF with ~50- and 20-fold higher K_d values (K_d = 11.0 and 3.7 nM), respectively, than the wild-type receptor (Figures 4 and 6). The deletion either in the N-terminal half or in the entire region of the CRH domain (mutants Y and W) resulted in total inability to bind G-CSF. These results indicate that the N-terminal region of the CRH domain plays an essential role in the binding of G-CSF.

The CRH domain and part of the cytoplasmic region are necessary for signal transduction

We have then examined whether mutated G-CSF receptors are able to transduce the G-CSF-triggered growth signal into cells. Figure 7A shows the G-CSF dependent [^{3}H]thymidine uptake of the transformants expressing the mutated G-CSF receptor. Clones of FD-A and FD-E responded to G-CSF in a concentration-dependent manner, although the maximum response of FD-E clones was lower than that obtained with the wild-type G-CSF receptor. Clones of FD-A and FD-E could be maintained in the medium containing G-CSF instead of IL-3 (Figure 7B). On the other hand, G-CSF neither stimulated the DNA synthesis of FD-P and FD-X clones nor supported their long-term growth (Figure 7A and B). These results suggest that the 99 amino acid portion from position 626 to 724 in the cytoplasmic region is essential for transduction of the G-CSF-triggered growth signal into FDC-P1 cells. FD11 clones expressing the molecule which contains a 27 amino acid insertion responded weakly to G-CSF, and were able to grow slowly in the medium containing G-CSF.

G-CSF weakly stimulated [^{3}H]thymidine uptake of FD-7, FD-C and FD-G clones (Figure 7A) and these clones could be maintained in the medium containing G-CSF (Figure 7B), suggesting that neither Ig-like nor FNIII domains are involved directly in signal transduction. As expected from the total inability of the mutants W and Y to bind G-CSF, these receptors could not respond to G-CSF at all. It may be noteworthy that a weak [^{3}H]thymidine uptake independent of G-CSF and IL-3 was observed in some clones of FD-C, FD-G and FD-Y cells (Figure 7A). Interestingly, mutant 17, which contained a deletion in the C-terminal half of the CRH domain, was completely inactive in signal transduction even in the presence of 50 nM G-CSF (Figure 7A), which should be sufficient for the binding of G-CSF to this mutant (Figures 4 and 6). These results suggest that the CRH domain of the receptor plays an essential role in signal transduction by G-CSF.

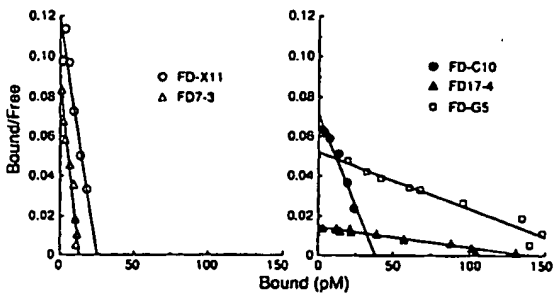


Fig. 6. Scatchard analysis of mutant G-CSF receptors expressed in FDC-P1 transformants. Cells of transformants FD-X11 (2.5×10^6 cells/ml), FD-C10, FD7-3, FD17-4 (10^7 cells/ml) and FD-G5 (2.7×10^7 cells/ml) were incubated with various concentrations of [^{125}I]G-CSF for 3 h at 4°C and specific binding was determined as described in Figure 1A.

Discussion

G-CSF receptor is able to function as a growth signal transducer in IL-3-dependent cells but not in IL-2-dependent cells

To explore the signal transduction mechanism of the newly identified cytokine receptor family (Bazan, 1990b), it is essential to introduce the receptor cDNAs into various cells, especially hemopoietic cells. However, the expression of cDNA in hemopoietic cells is not an easy task. In this report, we have successfully used the promoter of human EF-1α gene to express the G-CSF receptor cDNA in various hemopoietic cell lines (Figure 1). The expression level of the G-CSF receptor driven by the EF-1α promoter in murine FDC-P1 cells was ~100 times greater than that driven by a CMV promoter (unblished observation).

Expression of the G-CSF receptor in IL-3-dependent FDC-P1 or BAF-B03 cells enables these cells to grow in response to G-CSF (Figures 2 and 3). These results clearly indicate that the G-CSF receptor encoded by the cloned cDNA is sufficient to transduce the growth signal into cells, and suggest the presence of a common signal transducing pathway for the IL-3 and G-CSF systems. Introduction of protein kinases such as v-src, v-abl and v-fms into IL-3-dependent cells abrogated the dependence of cells on IL-3 (Cleveland *et al.*, 1989). Stimulation of cells with IL-3 induces the phosphorylation of tyrosine residues in a set of proteins, suggesting that some tyrosine kinase is involved in signal transduction by IL-3 (Koyasu *et al.*, 1987; Isfort *et al.*, 1988). Recently, Isfort and Ihle (1990) have shown that IL-3 and G-CSF stimulate tyrosine phosphorylation of the same protein (pp56) in NFS-60 cells. It is possible that the pp56 protein is one of the signal transducing molecules common in IL-3 and G-CSF systems.

Hatakeyama *et al.* (1989b) introduced the cDNA for the β-chain of IL-2 receptor into IL-3-dependent BAF-B03 cells. Since the BAF-B03 cells expressing IL-2 receptor could grow in the presence of IL-2, these authors postulated common signal transduction pathways in the IL-2 and IL-3 systems. However, G-CSF did not support the growth of the IL-2-dependent CTLL-2 cells transformed with the G-CSF receptor cDNA, though these cells expressed the high affinity G-CSF receptor in a large amount (Figure 1). These results may suggest that the signal transducing pathways downstream of the receptor are similar but different in the G-CSF and IL-2 systems, and that CTLL-2 cells are deficient in some components which are necessary for G-CSF-triggered signal transduction.

Homodimer and heterodimer of the cytokine receptor

As shown in Figure 8, recent analyses on the structure of cytokine receptors and reconstitution of the receptors using their respective cDNAs have revealed that the receptors for IL-6, IL-2 and GM-CSF consist of two different subunits (Hatakeyama *et al.*, 1989a; Hibi *et al.*, 1990; Hayashida *et al.*, 1990). The α-chain of each receptor binds its ligand with low affinity, and the second chain (β-chain) is necessary for formation of the high affinity binding site and for transduction of the signal. In contrast, the single polypeptide of the G-CSF receptor constituted a high affinity binding site for G-CSF, not only in hemopoietic cells (Figure 1A) but also in epithelial cells such as COS cells (Fukunaga *et al.*, 1990a,c) and C127I cells (unpublished observation). Previously, we have shown that the monomer of the purified G-CSF receptor has a low affinity for G-CSF, whereas its dimer or oligomer constitutes a high affinity binding site for G-CSF (Fukunaga *et al.*, 1990b). Since the dissociation

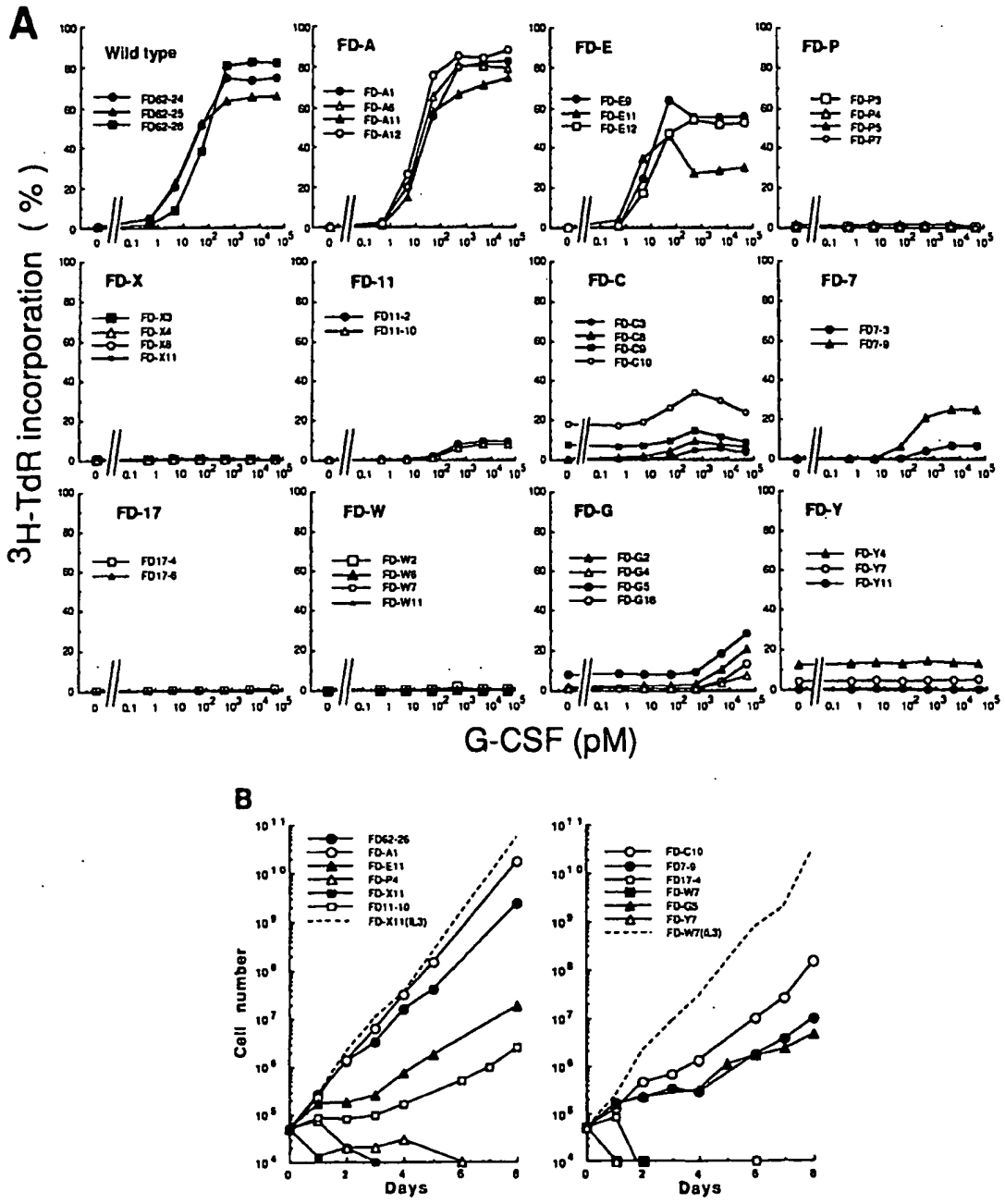


Fig. 7. G-CSF-dependent growth of transformants expressing mutant G-CSF receptor. (A) G-CSF-dependent [^{3}H]thymidine incorporation into cells. [^{3}H]thymidine incorporation was measured with several independent clones of individual FDC-P1 transformants expressing the mutant receptor as described in Figure 2. (B) G-CSF-dependent long-term proliferation of the transformants expressing mutant G-CSF receptor. Increase in cell number of FDC-P1 transformants expressing the mutant receptor in the presence of 20 nM (for FD-G transformant) or 500 pM (for other transformants) G-CSF was counted as described in Figure 3. Dotted lines indicate the growth curve of FD-X11 and FD-W7 cells in the presence of IL-3.

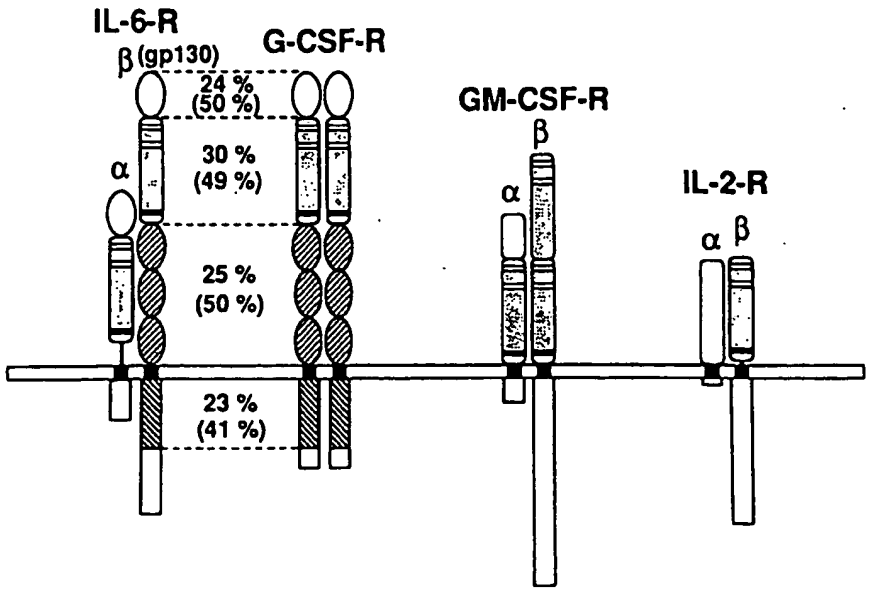


Fig. 8. Schematic representation of homo- and heterodimeric structures of functional, high affinity receptors for G-CSF, GM-CSF, IL-2 and IL-6. The CRH domains containing the conserved cysteine residues (thin bars) and the 'WSXWS' motif (thick bar) are indicated by shaded boxes. The gp130 protein (the β-chain of the IL-6 receptor) shows remarkable homology with the G-CSF receptor except for a C-terminal part of the cytoplasmic domain. The numbers indicate the percentage of identical amino acids in each subdomain. The numbers in parentheses show the percentage of homology including the conservative substitutions.

constant observed in intact cells is similar to that obtained with the dimer of the purified G-CSF receptor, it is likely that the G-CSF receptor exists as a dimer on the cell surface. Indeed, the cross-linking of the receptor with [^{125}I]G-CSF suggested the existence of a dimer of the receptor (Figure 1C). Availability of the antibody against the G-CSF receptor will make it possible to clarify the mechanism of the receptor dimerization in more detail.

Ligand binding domain of G-CSF receptor

Since the IL-4, IL-7 and erythropoietin receptors and the β-chain for the IL-2 receptor contain only the CRH domain in the extracellular region, it was postulated that this domain is involved in binding of the ligand (Bazan, 1990b). The present study using deletion mutants of the G-CSF receptor agrees with the above hypothesis. The receptor deleted in the C-terminal half of the CRH domain (mutant 17) could bind G-CSF with low affinity, while the mutants lacking the N-terminal region of the CRH domain (mutants W and Y) completely lacked the ability to bind G-CSF, suggesting that the N-terminal half containing the four conserved cysteine residues is indispensable for binding the ligand. Recently, Bass *et al.* (1991) have also shown that the hormone binding determinants of the growth hormone receptor is in the cysteine-rich region of the CRH domain.

The fact that mutants G and 17 bound G-CSF with low affinities suggests that the Ig-like domain and/or the C-terminal portion of the CRH domain may participate in the recognition of G-CSF, although it is possible that deletions in these domains simply caused some steric hindrance or conformational change in the ligand-binding site. An alternative possibility is that these domains are responsible for dimerization of the G-CSF receptor. The latter possibility may explain why these mutants showed low affinity binding to G-CSF with K_d values (3.7 and 11 nM) similar to those observed with the monomeric protein of the purified G-CSF receptor (2.6–4.2 nM, Fukunaga *et al.*, 1990b). No cytokines showed a significant homology to G-CSF except for IL-6, which has a limited similarity to G-CSF (Nagata, 1990). Nevertheless, the CRH domain, which is the ligand-binding domain of the cytokine receptor molecule, showed a significant homology among these cytokines (Bazan, 1990b). Bazan (1990a) has recently proposed, based on the extrapolation from their primary structures, that these cytokines may have similar tertiary structure. Detailed mutational analysis of the ligand and the CRH domain, as carried out with growth hormone (Cunningham and Wells, 1989; Bass *et al.*, 1991), may reveal the mechanism through which different cytokines specifically recognize their own receptor.

Signal transducing domain of the G-CSF receptor

In the two mutants showing low affinity binding to G-CSF, the mutant G could respond to high concentrations of G-CSF, whereas the mutant 17 was totally inactive (Figures 4, 6 and 7). This result suggests that the C-terminal region of the CRH domain is indispensable for the signal transduction triggered by G-CSF. This C-terminal region, which contains the 'WSXWS' motif, may play a role in transducing some conformational change induced by the binding of G-CSF across the membrane to the cytoplasmic domain of the molecule. Alternatively, if the region deleted in the mutant 17 is involved in the formation of the dimeric receptor as discussed above, this result may imply that dimerization of the G-CSF receptor has a significance in signal transduction, as suggested in the cases of PDGF and EGF receptors (Heldin *et al.*, 1989; Bishayee *et al.*, 1989; Spaargaren *et al.*, 1991). In the [^{3}H]thymidine incorporation assay, the transformants expressing mutants G, C and 7 responded weakly to G-CSF (Figure 7A) but were able to grow slowly in the presence of G-CSF (Figure 7B). The Ig-like and FNIII domains, therefore, do not seem to play

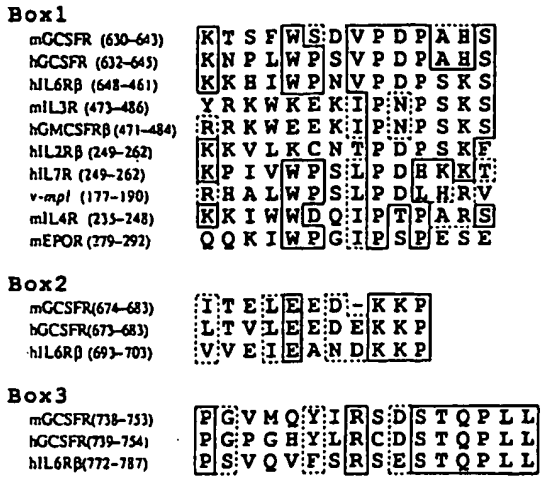


Fig. 9. The amino acid sequences conserved in the cytoplasmic domains of the G-CSF receptor and the β-chain (gp130) of the IL-6 receptor. The amino acid sequences of the corresponding regions of murine G-CSF receptor (mGCSFR, Fukunaga *et al.*, 1990a), human G-CSF receptor (hGCSFR, Fukunaga *et al.*, 1990c) and the β-chain of human IL-6 receptor (gp130) (hIL6Rβ, Hibi *et al.*, 1990) are aligned. The amino acid sequence of the Box 1 region of murine IL-3 receptor (Itoh *et al.*, 1990), the β-chain of human GM-CSFR (Hayashida *et al.*, 1990), the β-chain of human IL-2 receptor (Hatakeyama *et al.*, 1989a), human IL-7 receptor (Goodwin *et al.*, 1990), v-*mpl* (Souyri *et al.*, 1990), murine IL-4 receptor (Mosley *et al.*, 1989) and murine erythropoietin receptor (D'Andrea *et al.*, 1989) are also shown.

essential roles in signal transduction. Binding experiments using [^{125}I]G-CSF and Western blotting analysis have indicated that the numbers of the receptor expressed in these cells are lower than those in cells expressing the wild-type receptor (Figures 4 and 5). These results suggest that deletion of the FNIII domains may render the molecule unstable. The low response of these cells to G-CSF may be partly due to the poor expression of these mutant proteins.

The progressive deletion of the cytoplasmic region of the G-CSF receptor identified a region of 99 amino acids (positions 626–724) which seems to be essential for signal transduction by G-CSF in FDC-P1 cells. This region may constitute a domain of an unknown enzyme activity or associate with other molecules which transduce the signal. Previously, we have noticed that the cytoplasmic region of the G-CSF receptor has a similarity to that of the IL-4 receptor (Fukunaga *et al.*, 1990a). However, the homology between the cytoplasmic regions of the G-CSF receptor and the newly isolated gp130 (the β-chain) of the IL-6 receptor (Hibi *et al.*, 1990) is much more pronounced (Figure 8). As shown in Figure 9, three stretches of amino acid sequences (Boxes 1–3) are highly conserved between the G-CSF receptor and gp130, and two of the sequences (Boxes 1 and 2) are in the region which is critical for the signal transduction of the G-CSF receptor. IL-6 works on various cells including B cells, T cells and myeloid cells, and the gp130 seems to be responsible for the signal transduction triggered by IL-6 (Hibi *et al.*, 1990). The conserved region identified above may also be involved in signal transduction through the gp130 in the IL-6 system. In this regard, it may be noteworthy that the sequences similar to 'Box 1' can be found in the cytoplasmic region of receptors for IL-3, GM-CSF, IL-2, IL-7, IL-4 and erythropoietin, and in a recently identified oncogene v-*mpl* (Figure 9). In addition to the stimulation of cell proliferation, G-CSF and IL-6 have an ability to induce cells to differentiate. Whether or not the region involved in transducing the differentiation signal is identical to the region for the growth signal remains to be studied.

Materials and methods

Cells and cell culture

Murine myeloid leukemia cell lines, NFS-60 cells (Weinstein *et al.*, 1986) and FDC-P1 cells (Dexter *et al.*, 1980) were kindly provided by Dr J.N.Ihle (St Jude Children's Research Hospital). Mouse pro-B cell line, BAF-B03 (Hatakeyama *et al.*, 1989b) and mouse cytotoxic T cell line, CTLL-2 (ATCC TIB 214) were provided by Drs M.Hatakeyama and T.Taniguchi (Institute for Molecular and Cellular Biology, Osaka University). NFS-60, FDC-P1 and BAF-B03 cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, Hyclone) and 10–20 U/ml of recombinant mouse IL-3 (Fukunaga *et al.*, 1990b). CTLL-2 cells were grown in RPMI1640 medium containing 10% FCS and 10 ng/ml recombinant human IL-2 which was kindly provided by Dr H.Matsui (Ajinomoto Co., Tokyo).

Plasmid construction

Plasmid pI62 carrying the full length cDNA for mouse G-CSF receptor in CDM8 vector has been described previously (Fukunaga *et al.*, 1990a). Two variant cDNAs, pG17 and pF1, were obtained from the NFS-60 cDNA library (Fukunaga *et al.*, 1990a) by colony hybridization with the cDNA of pI62 as a probe, and will be described elsewhere in detail. The *Xba*I cDNA fragment was excised from pI62, pG17 and pF1, and inserted into the *Xba*I site of a mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990) to produce pBOS-I62 (wild-type receptor), pBOS-G17 (variant 17) and pBOS-JF7 (variant 7). The expression plasmid for the wild-type human G-CSF receptor (pHQ3) and its variant 11 (pQW11) has been described previously (Fukunaga *et al.*, 1990c).

For the construction of mutants containing the deletion in the cytoplasmic domain of mouse G-CSF receptor, pI62 was digested with either *Xmn*I (at 2140), *Bsp*HI (at 2209), *Bst*EII (at 2421) or *Apa*I (at 2516), and if necessary, ends were blunted with the Klenow fragment of *E.coli* DNA polymerase I or T4 DNA polymerase. An *Xba*I linker (CTCTAGAG), which allows the addition of a leucine residue followed by an in-frame termination codon, was ligated to the blunt-ended DNA and digested with *Xba*I. The *Xba*I fragments containing the G-CSF receptor cDNA were then inserted into pEF-BOS to generate pBOSdXmn (mutant X), pBOSdBsp (mutant P), pBOSdBstE (mutant E), and pBOSdApa (mutant A).

To construct pBOSdWS (mutant W), pBOS-I62 was digested with *Kpn*I (at 1829) and *Bgl*II (at 358), and the *Bgl*II–*Kpn*I DNA fragment derived from the cDNA was digested with *Xho*II (at 1223 and 1288). The *Xho*II–*Kpn*I DNA fragment was separated using agarose gel electrophoresis and inserted into pBOS-I62, which was digested with *Kpn*I (at 1829) and partially digested with *Bgl*II (at 358). For the construction of pBOSdCON (mutant C), pI62 was digested with *Eco*T22I (at 1106) and *Bsp*HI (at 2209), and the *Eco*T22I–*Bsp*HI cDNA fragment was digested with *Xho*II (at 1223, 1288 and 2045). The 117 bp *Eco*T22I–*Xho*II and the 164 bp *Xho*II–*Bsp*HI DNA fragments were then ligated with pI62 digested with *Eco*T22I and *Bsp*HI. The *Xba*I DNA fragment of the resultant plasmid was inserted into pEF-BOS to produce pBOSdCON. To construct pBOSdIg (mutant G), the cDNA fragment of pI62 was digested with *Taq*I (at 265, 838 and 2000), treated with Klenow fragment and digested with *Hin*dIII (at 162) to yield a 103 bp *Hin*dIII–*Taq*I(blunt) fragment. In addition to this fragment, the cDNA fragment of pI62 was digested with *Tth*111I (at 297, 501 and 1938), blunt-ended and digested with *Kpn*I (at 1829). The 103 bp *Hin*dIII–*Taq*I(blunt) fragment and the 1.33 kb *Tth*111I(blunt)–*Kpn*I fragment were ligated together with a 6.3 kb *Hin*dIII–*Kpn*I fragment, which was prepared by digestion of pBOS-I62 with *Kpn*I (at 1829) and *Hin*dIII (partially at 162) to produce pBOSdIg. To construct pBOSdICy (mutant Y), the cDNA fragment of pI62 was digested with *Taq*I (at 265, 838 and 2000), *Hin*dIII (at 162) and *Kpn*I (at 1829); the 103 bp *Hin*dIII–*Taq*I fragment and 991 bp *Taq*I–*Kpn*I fragment were then ligated with the 6.3 kb *Hin*dIII–*Kpn*I fragment described above. All constructions were confirmed by restriction enzyme mapping and DNA sequencing analysis.

Transfection of DNA

Cells were transfected with plasmid DNAs by electroporation (Potter *et al.*, 1984). The electroporation was carried out using Gene Pulser (BioRad) essentially according to the manufacturer's instructions. In brief, 8×10^6 cells were suspended in 0.8 ml of phosphate-buffered sucrose [7 mM sodium phosphate buffer (pH 7.4), 270 mM sucrose and 1 mM $MgCl_2$]. Eighty micrograms of the G-CSF receptor expression plasmid which had been

linearized by digestion with *Apa*LI, and 2 μg of *Xho*I-digested pSTneoB (Katoh *et al.*, 1987) were added to the cell suspension, which was incubated on ice for 10 min. Cells were exposed to a 350 V pulse with a capacitance of 25 μF, and returned to ice. After incubation on ice for 10 min, cells were diluted with 50 ml of RPMI 1640 medium/10% FCS containing IL-3 or IL-2, and cultivated in 24-well plates. Transfected cells were selected by culturing cells in medium containing G-418 at a final concentration of 0.5 mg/ml (for FDC-P1, and CTLL-2) or 2 mg/ml (for BAF-B03). Subcloning of the transfected cells was carried out by limiting dilution.

Cell proliferation assay, binding of G-CSF and chemical cross-linking

1.5×10^4 cells (100 μl) were mixed with various concentrations of G-CSF, IL-3 or IL-2 in 96-well microtiter plates. After incubation at 37°C for 22 h, 0.5 μCi of [^{3}H]thymidine (specific activity, 74 GBq/mmol) was added per well and further incubated for 4 h at 37°C prior to harvest.

Radioiodination of murine recombinant G-CSF, binding of [^{125}I]G-CSF to cells, and chemical cross-linking were performed as described previously (Fukunaga *et al.*, 1990a,b).

Preparation of antibodies against murine G-CSF receptor

Two different anti-mouse G-CSF receptor sera (anti-MR1 and anti-MR9) recognizing either a portion of the extracellular domain (amino acid positions 36–326) or the cytoplasmic domain (positions 631–812) were prepared as follows. The corresponding regions of murine G-CSF receptor were first produced in *E.coli* using the expression system developed by Studier *et al.* (1990). The expression vector pGEMEX-1 (Promega) was digested with *Nhe*I and *Bam*HI and the ends were filled in using the Klenow fragment. The DNA was religated to generate pEX, in which the *phi10* gene was deleted and the *Bam*HI site was placed immediately downstream of an ATG initiation codon. To express the extracellular portions of murine G-CSF receptor, the 865 bp *Xho*II DNA fragment (nucleotide position 358–1223) of pI62 was inserted into the *Bam*HI site of pEX to generate pEX-MR1. To express the cytoplasmic region of murine G-CSF receptor, pGEX-MR9 was constructed by ligating the 900 bp *Xmn*I–*Sph*I DNA fragment (nucleotides 2140–3126) of pJ17 (Fukunaga *et al.*, 1990a) with the *Hinc*II- and *Sph*I-digested pGEMEX-1 vector.

E.coli BL21(DE3)pLysS (Studier *et al.*, 1990) was transformed with pEX-MR1 or pGEX-MR9, and the relevant products were purified essentially according to the method described by Sambrook *et al.* (1989). When the cell lysates were centrifuged, most of the recombinant proteins were found in inclusion bodies of the precipitate. After successive washing with H_2O and 0.1 M Tris–HCl (pH 8.5) containing 2 M urea, the precipitates were dissolved in a solubilizing buffer [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl containing 8 M urea and 0.1 mM APMSF]. The G-CSF receptor polypeptides were purified by gel filtration on an AcA 54 column which was equilibrated with the solubilizing buffer, and dialyzed against phosphate-buffered saline (PBS). The purified proteins, which were >90% pure, were used to immunize rabbits to obtain the antisera.

Immunoblot analysis

About 2×10^7 cells collected by centrifugation were suspended in 100 μl of PBS containing a mixture of protease inhibitors (Fukunaga *et al.*, 1990b), lysed by adding an equal vol of 2× sample buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol] and sonicated to shear genomic DNA. After heating at 95°C for 5 min, the samples were electrophoresed on a 4–20% gradient polyacrylamide gel. Electroblotting of proteins to a GVHP membrane filter (Millipore) was performed as described previously (Fukunaga *et al.*, 1990b). The blotted filter was rinsed with Block Ace (Dainippon Seiyaku, Co., Japan) for 60 min at 37°C, washed three times with PBS and once with TPBS (PBS containing 0.1% Tween 20), and incubated with 10 ml of TPBS containing 10% Block Ace and 1 μl of anti-MR1 serum or 0.1 μl of anti-MR9 serum for 60 min at room temperature. The filter was then washed three times with TPBS and incubated with 10 ml of TPBS containing 10% Block Ace and 18.5 kBq/ml ^{125}I-labeled F(ab')$_2$ fragment of donkey anti-rabbit Ig antibody (Amersham). After incubation for 60 min at room temperature, the filter was washed six times for 10 min each with TPBS, dried and subjected to autoradiography.

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